Evolution of cross-tolerance to metals in yeast

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Abstract

In changing environments, organisms are challenged by multiple selective pressures simultaneously. For example, mine tailings contain multiple heavy metal contaminants in the soil. To unravel the potential for cross-tolerance, we used *Saccharomyces cerevisiae* adapting to high concentrations of single metals in a short-term evolution experiment. We then tested our metal-adapted lines into every metal and measured their cross-tolerance. We found that there are many ways to evolve tolerance, with the exception of copper-evolved lines with very parallel fitness profiles and few mutations. The cross-tolerance between pairs of metals is not reciprocal and trade-offs evolved in specialized lines. Generalist phenotypes were more likely to evolve in environments where any metal mutations are not useful or a low-quality environment, while environments more receptive to any type of metal mutation gave rise to less cross-tolerant phenotypes. To understand the genetic basis of the metal adaptation and cross-tolerance, we sequenced the genomes of 110 yeast lines from our short-term evolution experiment. We found that ion transport and plasma membrane were respectively biological process and cellular component significantly over-represented GO terms. We hypothesized that environmental distance could predict the amount of cross-tolerance, so we evaluated different measures of distance among metal environments against our phenotypic data. We used (i) the oxidative-reductive potential of the metal evolution environment, (ii) the correlation of metal pairs in the soil, (iii) concordance in the effect of known metal resistance genes in yeast, and (iv) the amount of the metal in the ancestral media as a proxy for how much exposure the yeast has to each metal. We did not find a significant relationship with any of our predictors. Our results evolutionary stochasticity plays big role in cross-tolerance and that metal adaptations are idiosyncratic.

Author summary

Studying tolerance and cross-tolerance to stressful environmental conditions as traits of organisms becomes increasingly important in a changing world. To unravel the potential for cross-tolerance, we evolved *Saccharomyces cerevisiae* in lethal concentrations of single metals in a short-term evolution experiment. We matched each evolved line's mutation with the amount of cross-tolerance by testing each evolved line in every metal. We found trade-offs evolved and that cross-tolerance is not reciprocal on average for pairs of environments. Many different fitness profiles evolved in any one environment, with the exception of copper-evolved lines showing remarkable parallelism. We learned

that within the realm of stressful environments, generalist mutations arise in low quality environments and specialist mutations in highly receptive environments. Genes under selection involved in ion transport in the plasma membrane were significantly over-represented, however, many different genes influence the amount of cross-tolerance for metals. We hypothesized that the similarity in the environment would predict the amount of cross-tolerance, but the idiosyncratic nature of cross-tolerance to metals poses a challenge to predicting whether populations are likely to adapt or die when facing environmental stressors.

Introduction

Organisms evolved in their environments accumulate pleiotropic effects that remain unrealized and mostly untested [1], however understanding how cross-tolerance and cross-intolerance arise and are associated with genetic make-up or the environment itself could become a powerful predictive tool in a changing world.

Metals are responsible for a large part of soil pollution on earth [2]. The impact of heavy metals on organisms include a multitude of stressors that affect different physiological responses and can be lethal [3]. Because of their toxicity, metals are also frequently used in microbial control (e.g., in wine production), exposing directly and purposefully microbes to metals at elevated concentrations [4]. Industrialization over the past century has led to a massive increase in the use and release of heavy metals into the environment [5]. These pollutants, especially in the cases of lead and mercury, have been recognized for their directly negative effects on human health, and efforts have been put in place to reduce their rates of release into the environment [6, 7]. However, many metals are currently and continuously entering the biosphere via mining, smelting, leaching from plastics and electronics in landfills, agricultural runoff, automobiles, and roadwork [5]. Metals produce reactive oxygen species (ROS), which have the potential to damage membranes, DNA, and proteins, becoming toxic to cells when concentrations reach high levels [5]. Evolution of metal resistance can be very fast, and the selective pressure is on several gene pathways and physiological mechanisms [8–10]. For example, strains of *E. coli* evolved in silver environments show a variety of mutations impacting various physiological mechanisms and gene pathways [8]. Evolving yeast in a copper environment has yielded similar results where mutations were found in several gene pathways [9]. In non-model pine-associated fungus *Suillus luteus* growing on a zinc smelter site has shown that selection for heavy metal tolerance targets many gene pathways and types of genes involved in tolerance [10].

Several metals, such as iron, zinc, cobalt, nickel, copper, and manganese, act as micronutrients (essential in trace amounts for proper cell functioning and found in lab standard yeast growth media) in the eukaryotic cell but are toxic at high concentrations [11]. Micronutrients cannot be completely excluded from cells and fine-tuned regulatory systems ensure balance between heavy metal excess and deficiency. Yeast have evolved mechanisms for the uptake and use of these micronutrients (i.e., chelators, transporters, and antioxidants), and adaptation to an excess of these metal ions cannot simply exclude the metals from the cell [12]. Different metals induce slightly different chemical reactions and physiological responses, but some metals may be more similar to each other from the cell's perspective. For example, the same pathway may be involved in the response to an excess of more than one metals, such as protection by the metallothionein *CUP*, which binds to both copper and cadmium [13]. Conversely, the cell might deal with different metals using completely different mechanisms.

We used evolutionary rescue experiments in which populations of yeast were exposed to lethal doses of five essential metal co-factors (cobalt, copper, manganese, nickel, and zinc) and one toxic metal (cadmium). Only populations harboring sufficiently

large-effect adaptive alleles were able to grow in the highly toxic environment. By studying these adapted lines, we seek to understand correlated adaptations to different metals, revealing common routes for evolution in harsh metal environments. We assay how often cross-tolerance arises, where mutations improving fitness in one metal cause an increased or decreased fitness in the presence of a different metal (Fig 1).

Fig 1. Diagram of experimental design for evolution experiment and fitness tests. A- Two–hundred and forty single colony isolates of strain W303 are inoculated into liquid YPAD in three 96-well plates, yielding 240 ancestral lines. B- The 240 ancestral lines are exposed to a lethal dose of $CdSO_4$, $CoSO_4$, $CuSO_4$, $MnCl_2$, $NiSO_4$, and $ZnSO_4$, prompting evolutionary rescue, and yielding between 15 and 23 evolved lines for each metal. C- The growth rates of all evolved lines are measured in each metal environment using a Bioscreen C automatic plate reader.

Materials and methods

Strains and Media

Yeast populations used in this experiment are derived from haploid lines of the lab strain W303. A sample of frozen W303 stock was inoculated in liquid rich medium (YPD (RPI Research Products Y20090) + adenine, referred to as YPAD onward) and grown overnight. A 1:1000 dilution was performed and spread on YAPD plates in order to bottleneck the initial population down to a single cell (one cell founding each replicate well). Single colonies were picked and inoculated in three deep 96-well plates (YAPD), allowed to grow overnight, and diluted for use in adaptive screens. A sample of each well was also isolated and frozen at -80℃ (400 μL culture added to 400 μL 30% glycerol) to be able to confirm that the single-cell ancestor did not carry the adaptive mutation observed in that line (by growth assays and/or PCR). A whole population sample of W303 is used initially to measure growth rate in increasing concentrations of metal to obtain a tolerance profile and determine concentrations of each metal beyond which growth is not typically observed. The tolerance profiles were obtained using a Bioscreen C, a microbiology workstation that automatically tracks growth trajectories by measuring turbidity at regular intervals using wide band filters (Oy Growth Curves Ab Ltd., 2009). Using a gradient of increasing concentrations, the lowest concentration that conferred no growth was determined and recorded for use in evolutionary rescue experiments. See S1 Table for concentrations in mM of metals used for the evolutionary rescue experiment.

We performed tests on lines evolved from other experiments. We chose ten random petite and ten random grande lines from and outcrossing experiment (no metal selection) to see if the petite phenotype helped in specific metals. We used Copper Beneficial Mutation (CBM) lines from Gerstein et al (2015) to test if whole-chromosome duplications helped cross-tolerance to any metal.

Experimental metal concentration selection method

Shape of tolerance curves were drawn for the derived W303 and BY4741. Derived W303 single colony cultures were diluted 1:11 and grown overnight in 96 well plates. These lines were used to test the yeast's maximum growth rate in metal compounds at different concentrations (mM) to determine the lowest concentration of no growth (see S1 Fig). Further testing on smaller intervals in concentrations around the observed point of no growth, narrowed down the concentrations we chose for the experiment.

Evolutionary rescue experimental design

We prepared the yeast lines for experimental evolution by growing the lab derived yeast strain W303 in liquid YPAD overnight at 30℃. The culture was then plated onto YPAD agar plates and incubated at 30℃for 2 days. We picked single colonies from the agar plates and inoculated them into three 96 well plates containing 1 mL of YPAD in each well and incubated them overnight at 30℃. We measured the optical density of each of the wells and normalized the culture concentrations by diluting all to the lowest concentration recorded. 300 μL of each well were frozen at -80℃ (300 μL culture added to 400 μL 30% glycerol) for storage. The diluted, initially isogenic single-colony isolates were inoculated into three 96-deepwell plates with lethal concentrations of each metal and shaken (200 rpm) at 30℃ for 14 days (240 replicates per metal). All boxes were checked daily by visual examination of the bottom of the wells. Growth was recorded when we saw settling of cells on the bottom of a well. Because this initial growth is often faint, we then allowed 24 hours to confirm growth, after which the well was manually mixed, and two tubes of 400 μL of culture in 15% glycerol were frozen at -80℃. In order to screen for genetic adaptations (as opposed to physiological or plastic responses), we started each evolved line from frozen in liquid YPAD and allowed them to grow for 2 or 3 days until growth could be observed. We then inoculated 10 μL of this culture into 1 mL YPAD + metal at the concentrations used in the evolution experiment (see Fig 1). Wells that did not exhibit growth within 4 days were eliminated from the experiment. We also tested each of the evolved lines for respiratory mutations by spot plating them onto glycerol and YPAD agar plates to check for petite phenotypes.

Fitness measurements and correlates

The metal-adapted lines were tested for fitness in YPAD and in each of the metal environments at concentrations determined to be lethal for the ancestor. We have observed that yeast adapted to a certain concentration of metal in the deep 96-well plates (1 mL per well) are not able to grow at the same concentration in the Bioscreen C plates (130 μL per well), as also seen by Gerstein et al. (2015), potentially due to differences in aeration. In order to determine lethal concentrations in the Bioscreen C plates, we randomly chose three adapted lines for each metal and tested them in five concentrations of that metal spanning the range between the last concentration of high growth in the tolerance profile and the concentration used in the evolutionary rescue experiments. The highest concentration that allowed growth in all three adapted lines was chosen for use in fitness measurement assays. Maximum growth rate as a proxy for fitness is measured using the Bioscreen C. Fitness of each metal-adapted line relative to the ancestor in each environment was calculated by taking the difference between the maximum growth rate of the evolved line and that of the ancestor strain.

DNA extractions, library prep and sequencing

We extracted DNA and sequenced the genomes of one-hundred and ten yeast lines. One-hundred and nine experimentally evolved, metal adapted yeast lines plus their ancestral strain were cultured in liquid YPAD media overnight in a 30°C shaking incubator. Cells were then rinsed and resuspended with 1 M sorbitol. Samples were incubated in a solution composed of 1 M sorbitol, 0.5 M EDTA, and Zymolyase (5U/μL, Zymo Research E1005) to break the cell walls, followed by incubation in a buffer (1 M Tris-HCl, 5 M NaCl, 10% SDS, 0.5 M EDTA, 100% Triton-X and double-distilled water) with Proteinase K (20mg/mL, Luna Nanotech GPK-020) to lyse the cells. Samples were purified with phenol-chloroform-isoamyl alcohol (25:24:1, Sigma-Aldrich 77617-100), followed by precipitation using 100% isopropanol and washing with 70% ethanol. DNA

pellets were air dried and then rehydrated in nuclease free water. After the pellets had fully dissolved, samples were then treated with RNAse (10 mg/mL, Thermo Scientific FEREN0531) to remove any RNA contamination. Genomic DNA samples were further purified with silica columns and a binding buffer (guanidine-HCl, Tween-20, isopropanol, double-distilled water) followed by elution with nuclease free water. The Qubit DsDNA Broad Range Assay Kit (Invitrogen Q32853) was used for quantification, following the standard protocol. Whole genomes were sequenced with Illumina technology on a NextSeq 550 System, 150-bp paired-end performed at the Sequencing + Bioinformatics Consortium at the University of British Columbia, Vancouver, British Columbia, Canada. The raw reads are available from the National Center for Biotechnology Information Short Read Archive ((SRA code)).

Sequence data analysis, variant calling and aneuploidy analysis

Raw reads were demultiplexed with bcl2fastq v2.20.0.422, without any modification and then cleaned with cutadapt using default settings for read quality and adapter removal. Paired end reads were mapped to the *Saccharomyces cerevisiae* reference genome S288C in BWA [14]. Each mapped genome was then converted to a sam file, sorted, converted to a bam file, and finally indexed in SAMTools [15]. We marked duplicate reads, estimated genome coverage, called variants for each individual and then combined all the variants in the experiment with GATK4 [16]. SNPs present in the ancestral strain were filtered out and removed, so the remaining variants were used for downstream analyses.

We extracted, cleaned and filtered genomic SNP information from the VCF in R using the tidyverse package [17]. We removed any site with an overall quality score less than 1000. Removed multiple alternates by removing anything with a "," in "ALT"). If site information started with ".:0,0", we replaced that string with "missing:missing:0". We extracted the depth of coverage (DP) from the site information into a new column. Then we extracted the genotype (GT) code into a new column and whenever the DP value was less than 5, we replaced the genotype with "low coverage". We remove any sites where where all lines have the same genotype (ancestral to W303) and sites shared with the ancestral line W303. After annotation we removed from the set intergenic sites, sites with no annotation, and sites in genes *FLO1* and *FLO9*. The remaining mutations were visually inspected by viewing bam files in IGV and filtered out if the coverage was low or if they were ancestral.

We calculated total depth of coverage for each chromosome to estimate and detect chromosomal aneuploidy. We created a pileup file for each yeast line indicating coverage for each site in bamTools pileup program. The files were then processed with a custom perl script which adds together the depth of coverage in windows of 1000 bp. We used the same script to estimate the coverage for the mitochondrial genome of the petite lines in windows of 100 bp. We estimated a relative copy-number of the *CUP* genes by following the "*in silico*" protocol [9]. The protocol uses the unix command "grep" to count the instances of *CUP1* and *CUP2* sequences compared to loci (*RIX1*, *DED81*, *DUR3*) on Chromosome VIII. We report estimates of CUP compared to the estimate copy-number of our sequenced ancestral line W303.

Statistical analysis

These assays will allow us to determine how adaptive mutations for each metal affect fitness in all the other metals, yielding a dataset that allows measurement of the pleiotropic effects of adaptive mutations to different metal environments *in vivo*. As a fitness measure, we use maximum mitotic growth rate obtained using a loess fit to the growth curves obtained from the optical density measurements from the Bioscreen C (see details in [9]).

Go term fisher test

In our overarching analysis, we predict different components of pleiotropy and we test explicitly which ones are contributing the most to pleiotropy in metal environments. We include in our general linearized model (GLM) the relative fitness in the test environment (maximum growth rate of adapted line minus maximum growth rate of ancestor), and as explanatory variables we used the proportion of genes with synergistic effects on metal tolerance, the difference in media electropotential, and the co-occurrence of metals in soils. The model was fit using a mixed effect model using the evolution environment, the test environment, their interaction, the interaction of evolution, test and their combination with the strain, and bioscreen plate and machine. We quantified the contribution of these random effects to see how evolutionary stochasticity (each single strain had different SNP mutations) and evolution or test environment played a role in cross-tolerance. We used the package *lme4* [18] to run the random effects model with this formula: 1 + log(relfit + 1) ORP + prop.syn + all_slope + log(ppm) + petite + (1 — E2) + (1 — E1) + (1 — E1:E2) + (1 — machine/plate) + (1 — strain:E2) + (1 — strain:E1) + (1 — strain:E1:E2)

Saccharomyces Genome Database

The objective of this analysis was to integrate the information held in this database about the yeast genomic make-up, and if it can be used to predict pleiotropic relationships of adaptive mutations in a system of closely related stressors, in this case, toxic concentrations of transition metal ions. In this analysis we predict pleiotropy from gene annotations in the Saccharomyces Genome Database (SGD) [19]. A database query for the observable trait "metal resistance" yields a table of annotations from classical genetics studies and systematic mutation sets with the effects of different mutations on the fitness of yeast in different metals. The data was manipulated in R to obtain a list of genes altering resistance for each metal; this first dataset did not include information on mutation type (knockout or overexpression) or mutation effect (increase or decrease in resistance). A correlation analysis was performed in R to obtain the phi-coefficient, a measure similar to the Pearson correlation coefficient, and its corresponding p-value (equation 19.61 in [20, 21]). A 2x2 contingency table was obtained as follows: A is the intersect of the gene lists for two metals (metal1 and metal2); B is the number of genes for metal1 that do not intersect with metal2; B is the number of genes for metal2 that do not intersect with metal1; D is the number of metal resistance genes that are not in the list for metal1 or metal2. The phi coefficient was then obtained using this formula: Φ = (AD-BC) / $\sqrt{((A+B)(C+D)(A+C)(B+D))}$. The χ^2 values for each metal pair were obtained through calculations in R, and significance was determined by calculating the p-value using the pchisq() function from the stats package in R. Further analysis was performed using SGD data that includes information about the sign of the mutational effect. The original SGD data was filtered such that each row contained a gene, its mutation information (null or overexpression), the metal associated, and its phenotype (increased or decreased resistance to that metal). Each row was assigned a "gene activity" of + or – depending on if the mutation information was described as null (knockout or loss of function) or overexpression, respectively. Each row was assigned a "direction of change" of + or – depending on if the phenotype was described as an increase or decrease in resistance, respectively. Each row is assigned a "sign product" of + if the product of these two signs is positive (i.e., gene activity and direction of change are the same) or – if the product is negative. If the sign product of a mutation is the same for two metals, the direction of pleiotropy is positive and negative if not. This scheme was applied to all lists of common genes for each metal pair, and the proportion of positive pleiotropy was calculated by dividing the number of mutations with positive pleiotropy by the total number of common genes. We therefore expect a linear, positive

relationship between the number of genes overlapping between metals and the fitness of evolved lines in test environments.

US Geological Survey measurements of metals in the environment

We expect that positive pleiotropy will occur more often for a locus conveying tolerance to pairs of metals that are commonly found together in natural environments, as a measure of that we used the correlation coefficient between pairs of metals in the environment. We downloaded data from the US Geological Survey, specifically, the National Geochemical Database (https://mrdata.usgs.gov/geochem/ accessed September 1, 2021). From the whole database we filtered out only the metals we used in our experiments. For our analysis, we used the values that were most consistently reported for all six metals of interest in the database as parts per million (ppm) and measured by Inductively Coupled Plasma (https://mrdata.usgs.gov/geochem/doc/icp40.htm). The values were under columns: CD_ICP40, CO_ICP40, CU_ICP40, MN_ICP40, NI_ICP40, ZN_ICP40. We log-transformed the data to run our analysis. Before log-transforming the data, we converted all negative values to zeros because zeros and negative values are both considered measurements below detection levels from spectrometry after acid dissolution (USGS, Further documentation is at http://mrdata.usgs.gov/geochem/doc/analysis.htm). We filtered all sites with values below detection level for all the metals. We ran the same analysis for subsets of data for mining sites and for water sediment. We filtered to find this relationships in mining areas by filtering the 1567 records with the word "mine" in the "contamination source" column and separately filtering the 1166 records have the word "sediment" in the "DESCRIPT" column. the data to show where heightened levels (significantly higher than background levels) of metals co-occur at the same site, then obtain frequencies for co-occurrences based on number of sites.

Metal reactivity

Metal ions have different oxidizing and reducing potentials in solution. Our third hypothesis was that positive pleiotropy will occur more often for pairs of metals that have more similar values of oxidizing and reducing potential in our growth media. To confirm the concentrations measured by mixing metal stocks and yeast growth media correspond with presence of ions in the solution, we quantified the amount of available ions in the filtered metal media by ICP-OES analysis. Five ml of each of the metal media solutions used for the experiment were filtered with a 0.22 μm filter and preserved with 2 drops of TraceMetal Grade Nitric Acid. Eh values quantify the potential of a medium to transfer electrons. We used this as a proxy for our measure of environmental stress and selective pressure on the yeast. Redox readings were recorded with a YSI Professional Series Instrument Pro Plus sonde equipped with Ag/AgCl reference electrodes calibrated by Zobell solution 4M KCl. We converted the ORP readings into Eh values by adding the offset voltage of 200mV. We then used the absolute difference between Eh value pairs to use as a prediction of the amount of cross-tolerance between pairs of metals, overall we expect that more similar environments should lead to more cross-tolerance.

Results

Evolutionary rescue, fitness profiles and cross-tolerance

We inferred the cross-tolerance and -intolerance effects of evolving in stressful single-metal environments in yeast by comparing growth rates across environments and whole-genome sequencing of 109 evolved yeast lines. To accomplish this goal, we adapted the methods of [9, 22], increasing the concentration of metals until growth is no longer reliably observed. Evolutionary rescue is then observed when a subset of yeast populations are able to grow and settle when inoculated in replicate populations within deep well plates. By first bottlenecking the populations down to a single cell, we ensure that the yeast populations are initially isogenic and must acquire adaptive mutations to overcome these highly stressful concentrations of metal. Physiological or plastic responses are typically not enough to rescue the population from extinction (verified by confirming tolerance after a period in rich media). In previous work with copper, evolutionary rescue was shown to involve multiple gene targets among independent populations [9]. Because the populations were bottlenecked by streaking on plates and picking a single colony 24 hours prior to inoculation, these alleles will arise by mutation from the single cell ancestor that established the yeast colony, either before or after exposure to the metal. Those cells with resistance to metal rise in frequency in the population and rescue that population from extinction, leading to appreciable sedimentation of cells in the wells (e.g., see [9]). Once the adapted lines were isolated, samples were frozen for subsequent genomic sequencing and fitness assays in all metals used in this study. Our goal was to obtain adaptive mutations to each metal, while avoiding the accumulation of non-adaptive mutations that would obscure the results (e.g., confusing linkage effects with cross-tolerance effects).

We evolved a total of 120 lines in 6 metals (S1 Table) and sequenced the genomes of 109 plus their ancestral strain. We tested each line in every other environment including the permissive ancestral media (YPD). We characterized fitness profiles for each of the lines ((Fig 2). Five of the six metals showed no particular common tolerance pattern across lines evolved in the same environment. An exception to this was the remarkable parallelism we found in copper lines that showed consistent and very similar fitness profiles across test metals ((Fig 2C).

Fig 2. Relative growth rates of evolved lines tested in different metal environments. A-F- Each graph shows the relative growth rates of lines from one evolution environment in each test environment. The colored lines connect dots representing a single evolved line tested in different environments and shading highlights the lines' performance in their evolution environment. A- Cadmium. B- Cobalt. C- Copper. D- Manganese. E- Nickel. F- Zinc. G- Relative growth of evolved lines grouped by test environment. H- Relative growth of evolved lines by test environment calculated relative to each line measured in their own evolution environment.

Cross-tolerance is not symmetrical. The order of exposure to a stressful metal will matter and the tolerance is not reciprocal. For example, our copper evolved lines perform well in manganese, however manganese evolved lines perform poorly in copper. Similarly, zinc evolved mutations are helpful in tolerating nickel, but nickel evolved mutations do not enhance performance in zinc.

Lines evolved in excess metal acquired mutations that are costly when there is no metal excess as seen when tested in permissive media ((Fig 2G). All lines performed equally or worse than their ancestor in the YPAD media suggesting a trade-off.

In addition to calculating the relative growth by using the performance of the ancestor in each environment, we also calculated the relative growth of the lines

compared to the performance of that same line in their own environment. This allowed us to highlight the environments evolving strongly specialized lines on average (e.g. copper and manganese lines) or more generalist lines on average (e.g. cobalt) and to highlight the more challenging environments ((Fig 2H). Compared to their performance in their own evolution metal environment, lines perform better in the permissive media on average, in (Fig 2H the trade-offs for more specialized lines are below the zero line (e.g. many copper and manganese evolved lines), contrasting the more generalist lines showing even better performance than in their own evolution environment by being on average above the zero line (e.g. cobalt and zinc evolved lines tested in other metals).

Environments permissive to any metal adaptation generate specialists. We calculated the amount of cross-tolerance per line by averaging the relative growth rate in all environments. If a genotype performs well in all environments it's highly cross-tolerant and a generalist, conversely, if a genotype performs poorly across environments it is specialized and locally adapted. We then quantified the quality of each metal environment by averaging the relative growth rate of all lines tested in each environment. In a permissive environment most yeast lines with a metal adaptation should perform well and in a challenging environment even metal adaptations will not help with growth. These quantities allowed us quantify some generalist vs. specialist trends (Fig 3). We found a significant negative linear relationship between how receptive the environment is to different metal mutations and how generalizable the mutations generated in that environment are, when we excluded lines tested in their evolution environment (S2 Table). This suggests that organisms evolved in toxic environments will be more or less cross tolerant depending on how benign or harsh the environment is overall. For example, the manganese environment is receptive to many kinds of metal adaptations, but yeast lines evolved in manganese are specialized for manganese and perform poorly in other environments. Conversely, cobalt-evolved lines are able to grow broadly across different metal environments, but growing in a cobalt environment is challenging for all metal evolved lines.

Fig 3. Specialist vs. generalist and environmental receptivity. Average performance by evolution environment gives us a value for the amount of cross-tolerance of the lines and where they fall on the generalist-specialist spectrum. Average performance by test environment gives us a value for how receptive an environment is for any one metal mutation. A- including the relative growth rate of lines tested in their evolution environment. B- excluding lines tested in their own evolution environment.

Genomic changes underlying cross-tolerance

We identified single-nucleotide polymorphisms (SNPs) and describe whole-chromosome duplication events likely associated with metal resistance. Our variant calling protocol initially found 10,358 SNPs and we proceeded to filter our SNPs further. We removed 727 sites with an overall quality score less than 1,000, 75 sites containing multiple alternatives, presumably because of alignment issues. We remove 6,696 sites where where all lines showed the same genotype, which we assume is ancestral to W303. We removed 308 sites where there are 50 or more lines with low coverage. We annotated the remaining 348 SNPs and further removed a total of 219 sites including: intergenic sites, sites with no annotation, and sites in genes *FLO1* and *FLO9* known to have alignment problems due to recently expanded gene families [9]. This filtering left 129 variants. We excluded from the downstream analysis line MnBM42 characterized by 72 unique SNPs. Manganese is known to cause mutagenesis in DNA copying and this line has mutations in DNA repair genes. Although interesting, this line will be investigated in a future study and is beyond the scope of this article. The remaining variants were visually

inspected in genome viewer (IGV) to confirm our confidence in the SNP call. We especially suspected SNPs found in seven genes that occurred in many of the lines: *AAD4*, *BSC1*, *DAN4*, *ENA5*, *MSS11*, *PIR3*, *TIR3*, and *TIR1*. Of these, only *MSS11* is reported in the SGD to have influence on metal resistance: knock out strain is reported to have decreased resistance to mercury, cadmium, and zinc [23]. We found a total of 34 genes with high-quality SNPs (Fig 4A and Fig S2).

Fig 4. Genes involved in metal tolerance and GO term analysis. A- Cellular localization of genes associated with SNPs. Asterisks (*) indicate genes that are localized in different cellular compartments. Color pie-charts indicate the evolution environment of the mutation. B,C- Black bars show the proportion of each category for the whole genome and colored bars show the proportion of that category in our experimental lines. Colors indicate the proportion of each evolution environment of the mutations. p-values are indicated by asterisks (*) and do not account for multiple testing, we report the uncorrected values. None were significant with a Bonferroni correction. B- Cellular component. C- Biological process.

We summarized the underlying genetic mechanisms for metal tolerance by running a GO term analysis for biological processes and cellular components. We uploaded the list of genes on the GOslim webpage (https://www.yeastgenome.org/goSlimMapper) with the setting 'select all terms'. Some genes did not map to the 'biological process' database (*NFT1*, *SYG1*, *PHO81*, *OCA4*) and some did not map to the 'cellular component' database (*YHC1*, *COG1*, *YEF1*, *TFG1*, *GCD2*, *RRP46*). We queried the SGD database for each of the unmapped genes and manually scored. The cellular component terms for *YHC1*, *TFG1*, *GCD2*, and *RRP46* was categorized as "nucleus", *COG1* was categorized as "Golgi apparatus", and *YEF1* was categorized as "unknown". We could not assign existing GO Slim biological process categories to the unmapped genes. We categorized PHO81 as "phosphate metabolism", *SYG1* as "signal transduction", *NFT1* as "macroautophagy", and *OCA4* as "unknown". To test if any of the GO terms were significantly over-represented in our mutants we performed Fisher Exact Tests to see if the count was different than what we expect in the whole genome (Fig 4C-D). Whole-genome counts of categories were given in the GO Slim results. We extracted the whole-genome counts for genes that had to be manually categorized from the page for each Gene Ontology Term on the SGD. The subheading for the Annotations table in this page contains the number of genes that map to this GO term in the genome. The whole-genome counts for "unknown" process and components were found on the *Saccharomyces cerevisiae* Genome Snapshot page on the SGD in the "Summary of Gene Ontology (GO) Annotations" (as of 17 May 2023). For cellular component only the 'plasma membrane' was significantly over-represented in our evolved lines. Three biological processes were significantly over-represented: 'ion transport', 'invasive growth in response to glucose limitation', and 'response to chemical'. However, none of the Fisher exact tests were significant accounting for multiple testing with a Bonferroni correction, in the figure we report the significant p-values without multiple testing (Fig 4B-C).

We estimated whole-chromosome duplications and found duplications across all metals except copper (Fig. 5A and S3 Fig). Whole-chromosome duplications were convergently evolved in nickel where four lines all had duplications in chromosome XIII and XIV or three cadmium evolved lines had duplications in chromosome II. We were limited in our ability to test if any instance of aneuploidy would enhance cross-tolerance. However, we tested copper-evolved lines from [9] in the metals we used in our experiment. Gerstein et al. [9], unlike us, found many whole-chromosome duplications in their copper evolved lines. In both experiments, however, aneuploidy did not show any signal of being beneficial for metal tolerance (see S3 Fig).

Fig 5. Whole-chromosome duplications and CUP coverage against performance in copper. A- Whole-chromosome duplication estimates. Estimates are scaled to the ancestral (W303) estimate to be able to compare across lines and evolution environments. B- *CUP* scaled coverage is a proxy for the number of copies of the gene. Only copper evolved lines show an increased number of *CUP* copies, however, we show that copper tolerance can be achieved in many ways and in lines evolved in all metals.

We found many avenues for metal resistance leading to more ways for cross-tolerance to unfold. Copper evolved lines showed the greatest parallelism in their fitness profiles (Fig 2C) and the least number of distinct mutations (Table 1 and Fig S2). We estimated the scaled coverage of the *CUP* gene and found that coverage, a proxy for increased copy-number, increased only in some copper lines Fig. 5B. surprisingly, we also detected the lowest *CUP* coverage in the rest of the copper lines. Five of the lines with an increased number of copies of *CUP* also had a mutation in gene *TFG1* (Fig S2), the largest subunit of *TFIIF* (Transcription Factor II) facilitating both transcription initiation and elongation of RNA polymerase II [19]. Many lines evolved in other metals than copper show growth in copper despite lacking the increased copy-number of *CUP* (Fig 5), showcasing how many roads can lead to metal tolerance.

Table 1. Number of Distinct Mutations by Evolution Metal

Metal	Total*	SNPs	Cytology¥	Cell†	Biological††
Cadmium	7	2	5	4	2
Cobalt	11	10	1	7	10
Copper	4	3	0 (+ *CUP*)	4	2
Manganese	11	8	3	15	11
Nickel	10	6	4	8	5
Zinc	12	8	4	9	10

*SNPs plus whole chromosome duplications † Cellular Component †† Biological Process ¥ Number of different chromosomes with a duplication event across evolved lines.

For most evolved lines we found at least some mutation that can explain the increased tolerance. There were 31 lines for which we could not confidently detect any genetic change (Fig S2). Lines without reliable mutations are not ones with lower cross-tolerance, more likely t explain their better performance in metals compared to their ancestor, they harbor mutations we did not detect or measure, for example, increased copy-number of other important metal-related genes or epigenetic changes.

Petite phenotype and loss of mitochondria

Yeast cells are able to forfeit the use of their mitochondrion thanks to their ability to live anaerobically. The loss of a functional mitochondrion is detected phenotypically by testing yeast in glycerol-based media where the petite (decayed mitochondrion) form cannot grow properly in absence of sugars to ferment. All the lines evolved in cobalt (16) also evolved the petite phenotype, 17 out of the 22 of the manganese evolved lines were petite, three petite lines in copper, and two in nickel. By looking at the effect of being petite in the different metals, our lines show a pattern where growth in manganese and cobalt is significantly improved by having the petite form. We hypothesized that perhaps being petite was adaptive in these metals, however, we quickly discounted this hypothesis by testing lines from a different experiment (Fig. 6A-B). Our metal-evolved lines in manganese and cobalt are showing a false pattern

where petite lines showed significant improved performance in those metals compared to grande (mitochondrion functional) phenotypes. This is due to the fact that in manganese cobalt-evolved lines (cross-tolerant) and manganese-evolved lines (specialized to manganese) have a strong advantage, and when tested in cobalt, all cobalt lines do marginally better than any other yeast. The petite phenotype therefore co-varies with other mutations that allow these lines to grow well in these two environments.

Fig 6. Petite performance in metals and mitochondrial genome coverage. A- Grande and petite performance in metals and YPD. B- Grande and petite performance in manganese, cobalt and YPD from an outcrossing experiment using the same ancestral strain W303. C- Mitochondrial genome coverage for petite lines with some remnant mitochondrial genome. All cobalt evolved lines and two copper evolved lines had complete loss of mitochondrial genomic material and are not shown in this graph.

We mapped the mitochondrial genomic reads and estimated their coverage to see if the mitochondrial genes had been lost entirely. Cobalt evolved lines had no trace of mitochondrial DNA, however, manganese, copper, and nickel petite lines showed loss of of mitochondrial genes but a remarkable increase in coverage of some breakpoint regions between respiratory genes (Fig. 6C).

Idiosyncratic nature of cross-tolerance and alternative predictions

In this study, we focused on adaptations to metals and tested explicit predictions and hypotheses for positive pleiotropic effects to unpack what are the components that may allow us to predict cross-tolerance. We tested four factors that may contribute to predicting the amount of cross-tolerance.

We investigated the predictability of adaptation and how genetic mutations interact in the genome to allow for adaptation to metals. Genetic information for yeast is extensive, with the *Saccharomyces* Genome Database (SGD) collating data from thousands of studies that describe the effects of mutations on resistance to different metals. We hypothesized that cross-tolerance will occur more often for pairs of metals that show more overall overlap in genes with the same direction effect.

Cross-tolerance could be facilitated by an organism having experienced a pair of stressors simultaneously in its evolutionary history. We approached this hypothesis by measuring a correlation coefficient for pairs of metals in soils found in the US Geological Survey. The correlation coefficient was our proxy for how often pairs of metals are found together with the hypothesis that the more often they are found together the more cross-tolerance will result.

In our experiment, we standardized as close as we could the amount of stress by the metal by choosing carefully our concentration to be at the level where we would not see growth. However, different metals will have different oxidative and reducing potentials in their ion form and we hypothesized that more similar electropotential profiles would lead to more cross-tolerance for pairs of metals. To this end, we measured the oxidative reductive potential (ORP) of our metal media and used the distance among those measurements to predict cross-tolerance.

Baker's yeast has long been evolved in laboratory yeast growth medium and, at least in this organism's recent evolutionary history, this is its ecology. We considered that perhaps specialized strategies will evolve more readily for metals that are more common in the growth medium and more generalist strategies will evolve in metals the yeast is less exposed to.

None of our predictions explained cross-tolerance, suggesting that evolution of cross-tolerance in single metal environments is idiosynchratic and explained by evolution and test environments and a large part of it is due to evolutionary stochasticity (Fig 7).

In our model, we quantified the contribution of the evolution and test environments and the contribution of evolutionary stochasticity. This way, we were able to quantify how much each of our 'random effects' contributed to the variance in the cross-tolerance (relative growth rate across environments). 34.7% of the variance was explained by our random effects and 9.8% of the variance by measurement error (see S3 Table).

Fig 7. Predictions for cross-tolerance to metals. A- Heatmap of Oxidative-Reductive Potential (ORP) similarity of the different metal media. B- Linear model of ORP similarity scores against relative growth rates. C- Heatmap of cross-tolerance predictions (Φ) based on gene overlap from the SGD knockout libraries. D- Linear model of gene overlap score (Φ) against relative growth rate. E- Heatmap of the co-occurrence of metals in soils from the US Geological Survey. F- Linear model of soil metal correlations against relative growth rate. G- Logged parts per million of each metal used in our experimental evolution recorded from the permissive, ancestral media (YPD) against cross-tolerance score. Dashed lines indicate statistically non-significant relationships.

Discussion

Ecological specialization and local adaptation in nature predict that cross-tolerance should mostly be antagonistic, with fitness trade-offs as the cost of an adaptive phenotype. Of course, in nature not everything is locally adapted and generalist phenotypes do evolve. Our approach using similar environments as our evolutionary backdrop allowed us confirm the presence of trade-offs in specialized yeast lines, and to extend our understanding of how generalist and specialist phenotypes tend to arise. Habitat heterogeneity is a major explanation for the evolution and maintenance of generalists [24, 25]. More specifically, if the extent of the environmental variation exceeds the capacity of the one adaptation to meet the challenges of that environment, a 'mixed' strategy will be selected for, leading to generalists [26]. Remold [27] has argued epistatic pleiotropy explains the evolution of both specialists and generalists, depending on their environment. Jerison et al.'s [28] results suggest that specialists in experimentally evolved populations of yeast will arise if the mutations that are costly in other environments are either more frequent or more beneficial than those that confer cross-tolerance. In our study, we find a significant relationship between the quality of the genotypes produced (more or less cross-tolerant) and the quality of the environment (more or less yeast lines are able to grow). One possibility is to add to environmental uncertainty or heterogeneity, the generalizability of the adaptation (genotype quality) and the receptivity of the environment (environment quality) have a negative linear relationship, and will lead to more or less specialized, locally adapted populations.

Jerison et al. [28] and Bono et al. [25] results and analyses suggest that there is a relationship between the amount of environmental similarity and the amount of cross-tolerance we should expect to evolve. More similar environments will have a higher likelihood of fixing mutations that work in both environments and as the similarity (e.g. physiochemical) declines, the fixing mutations will more likely have detrimental effects in the away environment. We followed this logic for our analysis attempting to predict these distance matrices (physiochemical, genetic overlap, co-occurrence, abundance) and relating them to cross-tolerance. We could not find predictive power. Jerison et al. [28] evolved yeast in different environments, high salt, low glucose and high temperatures with gradients and found the gradients to show more cross-tolerance, we may not have been able to detect this difference because the metal environments we chose are much more similar to one another. We also conclude that metal environments are

idiosyncratic and evolutionary stochasticity plays a large role in cross-tolerance. Even yeast populations that are evolved in the same stressful environment can accumulate different sets of mutations conferring more or less cross-tolerance [25, 28].

We predicted that the genes involved in metal tolerance were going to span the many strategies organisms evolve to cope with metal stress [8, 10, 12]. The Gene Ontology analysis of the genes with mutations we detected, suggested that the genes we found were mostly involved in transporter proteins in the plasma membrane. Transporters, membrane proteins mediating the delicate balance of metal homeostasis are known to be under selection from studies in yeast and other fungi [29–32]. Several mutations in our set are known transporters reported to be important in metal adaptations: *PMA1* [33–36], *PHO84* [37–39], *FCY2* [40], *OCA4* [41], and *YPK1* (also known as a potassium channel Tok1) [42].

Although transporters were the most represented genes in our suite of mutations, we also found mutations in genes that help with oxidative stress and repairing the damage from oxidative stress. For example, *SNT2* regulates gene expression in response to oxidative stress from peroxide. In this category is also *HUL5*, a cytosolic quality control protein [43] that evolved mutations in three cadmium lines with different whole-chromosome duplications, and therefore likely convergent.

Most mutations evolved in metals that do not match the metals in the literature where they were also reported, supporting the idea that many mutations can help in different metals. Our strains evolved *PTK2* in cobalt stress, but it was identified in a study looking at iron and copper overload [44]. A deletome analysis revealed that *SSK* is required for arsenic, cadmium and zinc tolerance [45] and evolved in cadmium environment in experimental evolution experiment [36]. However, in our study *SSK* evolved in manganese. Finally, *ACC1* mutations evolved in two zinc lines, hoever this gene was shown to aid cadmium tolerance acting downstream on a sulphur assimilation pathway [46].

Based on the many different ways metal tolerance can be achieved, we predicted we would find chelator proteins that bind metals and render them bio-unavailable. However, we did not find chelator proteins apart from increased copy-number of *CUP*, the copper binding protein [9, 47]. Increased copy-number in *CUP* was only in copper evolved lines, and in five of those lines, we also found a SNP mutation in the large subunit of TFIIF transcription factor *TFG1*. *TFG1* is a known suppressor of a mutant transcription factor TFIIB characterized by cold-sensitive growth defect [48]. Copper evolved lines showed a dramatic increase in fitness when tested in copper, more than other lines tested in their home environments. As evolutionary history shapes selection during subsequent adaptation steps [49], this could be due to the long history of using copper sulfites to discourage microbial growth leading to strategies for yeast to withstand high-concentrations of copper conditions [9, 47].

Whole chromosome duplications evolved in several metals and were showed parallelism with metals (e.g. chromosome XIII in nickel and zinc, chromosome II in cadmium and zinc). Gerstein et al. [9] found extensive aneuploidy of whole chromosomes in their copper-adapted lines. Gorter et al. [36] evolved yeast in cadmium and found whole genome aneuploidies and whole-chromosomes increase in chromosome XIV, III and VI, and decrease in ploidy in chromosome I. We found one mutation (*SMC2*) in a petite nickel evolved line involved in overseeing order and structure of chromosomes [50, 51].

A cellular process that was also identified as important was "invasive growth in response to glucode limitation", suggesting a prominent role of metabolic functions in metal tolerance. One of our manganese evolved lines (grande phenotype) gained a mutation in cytochrome c oxidase (*COX1*), which is found in the inner mitochondrial membrane. *COX1* reduces molecular oxygen by translocating four protons across the

membrane in the electron transport chain and it relies on copper, a critical redox cofactor. Many of our evolved lines, however, exhibited a petite phenotype and either complete loss or partial loss of mitochondrial genes. If the loss was partial, it always eliminated the respiration genes. [52–54]. The evolution of petite phenotypes is attributed to recombination events between repeats of GC clusters and AT stretches, which are common in non-coding mitochondrial genomes in yeast and especially in highly homologous origins of replication. Osman et al. [55] noticed that mtDNA excision events in petites tend to cluster near replication origins and show extensive structural variation, and Nunn and Goyal [56] showed how they coincide with breakpoints. We also found extensive structural variation and very high coverage of those non-coding regions in the mitochondria of petites without complete loss of the mitochondrion.

Among our manganese evolved lines we found MnBM_42 with 72 SNP mutations. This incredible number of mutations is likely due to manganese itself. Manganese has mutagenic effects on organisms by directly impacting DNA polymerase copying accuracy by replacing magnesium as a cofactor. When manganese replaces magnesium, the reduced specificity of insertion and excision leads to an increased mutation rate in *E. coli* and misincorporated deoxynucleotides [57]. We suspect this number of mutations has to do with low fidelity of the new DNA copy due to the replacement of manganese in the polymerase rather than being an adaptive strategy for metal tolerance.

Conclusion

Using experimental evolution of many lines evolved per metal environment and genomic information, we uncovered strategies of metal tolerance and the cross-tolerance for metals of each line. The various metal-adapted lines evolved tolerance and achieved cross-tolerance through different SNP mutations, chromosomal duplications, and copy-number variation. Our results show that fitness profiles can be variable through multiple evolutionary trajectories and different genomic changes or can repeatedly lead to similar cross-tolerance profiles, consistent with repeatable evolution. We also document the relationship between the amount of cross-tolerance and properties of the evolution environment, where more cross-tolerant lines will evolve on average in environments where not many mutations will work, but little cross-tolerance will evolved more in environments that are highly receptive to any metal-beneficial mutations. Cross-tolerance is idiosyncratic and while we hypothesized that the distance between pairs of metal environments measured in several ways would predict cross-tolerance, it did not, highlighting the critical role of stochasticity in the evolution of cross-tolerance.

Supporting information

S1 Fig. The Shape of Tolerance. Tolerance curves for incremental concentrations of metals of W303 (A-F) and BY4741 (G-L) genetic backgrounds recorded on the Bioscreen C optical density readers over three days measured every 15 minutes.

S2 Fig. Mutations and Cross Tolerance. Each mutation in each evolved line. The color of the heatmap boxes is determined by averaging the relative growth rate in all environments.

S3 Fig. Chromosome Ploidy Estimates. Each chromosome coverage estimate (1kb windows) for every evolved line.

S4 Fig. Evolved Line Performance and Whole Chromosome Duplications. We compared our results with a previous experiment with a different genetic background where the yeast was evolved in copper. Aneuploidy does not systematically lead to more cross tolerance to any metal.

S1 Appendix. Data Analyses.

S1 Table. Evolved lines. Number of lines evolved for each metal (out of the initial 240). Concentrations used in the evolution experiment in the deep-well plates were different than the concentrations used in the bioscreen assays by 15%. We also report the total number of petite phenotypes we found in each environment.

S2 Table. Linear Model of Environment vs. Genotype Quality. We quantified the quality of the environment by averaging the relative fitness of all the lines tested in that environment (excluding the lines evolved in that environment) and we quantified the quality of the genotype by averaging the relative fitness of each line in all test environments except their evolution environment.

S3 Table. ICC Random effect model statistics. Quantification of each random effect and the process behind the random effect.

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