Designing yeast as plant-like hyperaccumulators for heavy metals

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Hyperaccumulators typically refer to plants that absorb and tolerate elevated amounts of heavy metals. Due to their unique metal trafficking abilities, hyperaccumulators are promising candidates for bioremediation applications. However, compared to bacteria-based bioremediation systems, plant life cycle is long and growing conditions are difficult to maintain hindering their adoption. Herein, we combine the robust growth and engineerability of bacteria with the unique waste management mechanisms of plants by using a more tractable platform—the common baker’s yeast—to create plant-like hyperaccumulators. Through overexpression of metal transporters and engineering metal trafficking pathways, engineered yeast strains are able to sequester metals at concentrations 10–100 times more than established hyperaccumulator thresholds for chromium, arsenic, and cadmium. Strains are further engineered to be selective for either cadmium or strontium removal, specifically for radioactive Sr⁹⁰. Overall, this work presents a systematic approach for transforming yeast into metal hyperaccumulators that are as effective as their plant counterparts.
Heavy metal contamination is a growing environmental concern as the world becomes increasingly industrialized. Mining, manufacturing, and disposal of electronic goods are the main sources of heavy metal waste; the United States alone adds 262 million tonnes (289 tons) of waste per year to the growing 850 and more landfills. To illustrate the impact of waste generation this work specifically looked at two significant, yet often overlooked, contributors to heavy metal waste which are the textile industry and pollution from nuclear power plants and past fallout. Textile manufacturing employs a variety of heavy metal related processes, in particular dying, with many of the 100,000 types of dyes containing metal chelated centers for coloration. Particular regions, such as India and Bangladesh where textile manufacturing is a dominant industrial practice, see high levels of cadmium, chromium, and lead in soils which can reach 10–100 times higher than WHO established safety limits. Other metals such as cobalt, copper, zinc, and nickel are also pervasive and are incorporated at different levels in the textile process. The result, leachate that contains an indiscriminate mixture of metals which are difficult to separate, therefore leaving barriers of transport to renovate areas as the only viable waste management option. On the same vein, the problem of nuclear waste and past nuclear fallout, such as previous catastrophic events of Chernobyl and Fukushima, have refocused attention on radioactive metal contamination, specifically radioactive strontium (Sr\(^{90}\)) which is of particular interest for its biological implications in bone integration and cancer. However, given the molecular similarity of calcium and strontium, and the relative abundance of calcium over strontium, removing just Sr\(^{90}\) without being overwhelmed by other species is challenging. Both waste scenarios expose a unique challenge, how to selectively capture and discriminate metals from one another. Removal of toxic elements such as cadmium and mercury should be prioritized, even if at lower concentrations than more abundant and less harmful elements such as calcium and magnesium. This is particularly true for radioactive elements such as Sr\(^{90}\), where strontium is typically masked by large amounts of similar divalent metals like calcium. Current industrial approaches such as absorption and ion-exchange are not particularly effective for precise removal of toxic yet low concentration of heavy metals as these processes are first saturated by more abundant background metals.

Biores-mediation strategies have the potential to address the challenge of heavy metal contamination. A promising subset of bioremediation is phytoremediation, the use of plants to sequester pollutants from soils and water. Plants have developed mechanisms to uptake heavy metals without suffering major toxic effects, and their abundant and renewable biomass contribute to significant bioaccumulation of toxins from soils and waters. Out of all plants, there are more than 400 hyperaccumulator species; the stricter definition being an accumulation of 100 mg/kg of dry weight (DW) (0.01% DW) of cadmium or arsenic, 1000 mg/kg (0.1% DW) of cobalt, copper, chromium, aluminum, nickel, or lead, and 10,000 mg/kg (1% DW) of manganese, iron, or zinc. Not all hyperaccumulators have equal metal preferences. Even in a single family such as Brassicaceae, out of the 87 species 67 are nickel hyperaccumulators, 15 are zinc, and 5 can do both. The mechanism of hyperaccumulation have been attributed to hyperactive metal transporters and a variety of detoxification pathways which include glutathione synthesis and metal compartmentalization in vacuoles and other organelles.

What limits wide-spread adoption of plant-based remediation solutions is their maintenance and engineering complexity. Plants are complex organisms, with different species requiring strict growing conditions where hyperaccumulators found in one location may not necessarily thrive in others due to surrounding biotic and abiotic factors. More so, current phytoremediation technology takes weeks to years to see signs of remediation, and in this current global waste crisis may be too long of a time scale. There have been attempts to create transgenic plants which incorporate genes from hyperaccumulators which grow faster and are more resistant to environmental factors. However, because plants are multi-cellular with more complex gene clusters, the current state of genetic tools have yet to realize the sophistication and ease of engineering compared to their single-celled counterparts such as bacteria and yeast. Therefore, design of faster and easier waste management technologies need to be developed on other platforms that are more scalable and cost-effective. Single-cell organisms such as bacteria offer ease and scalability; however, they lack many hyperaccumulating features such as hyperactive metal transporters and useful organelles such as a vacuole. A biological platform at the intersection of these two is the common baker’s yeast, \textit{S. cerevisiae}. Current genetic engineering technologies have made it possible to engineer yeast on all levels, from specific proteins to complex metabolic pathways. More so, the infrastructure and ability to scale and distribute yeast are already in place thanks to the beer and pharmaceutical industries. The results herein show that taking concepts from plant hyperaccumulators and engineering them into yeast can generate similar hyperaccumulating capabilities that are equal or better than their plant-based counterparts.

**Results**

**Expressing metal transporters increase metal uptake.** Several fundamental metal trafficking components are essential for enhanced metal uptake in hyperaccumulating plants, including cell membrane transporters, organelles storage systems, and chelator molecules. (Fig. 1a). Metal ions enter via highly active membrane transporters, and are compartmentalized into organelles such as the vacuole. To limit cellular toxicity, chelators such as glutathione, metallothionein, and phytochelatins bind and remove metals from sensitive metabolic functions. To mimic these plant hyperaccumulating features, the first step was to identify and express a hyperactive membrane transporter. A set of membrane metal transporters for zinc, copper, iron, and manganese were overexpressed in yeast. For this study, native yeast metal transporters ZRT1 (accession number P32804), ZRT2 (Q12436), CTR1 (P49573), CTR3 (P06686), FTR1 (P40088), FET4 (P40988), SMF1 (P38925), and SMF2 (P38778) (ZRT3, CTR2, and SMF3 are vacuole transporters, while FET3 is an oxidoreductase) were cloned and overexpressed using a GAL1 promoter on a 2 microplasmid. When overexpressed, some of the transporters, along with several more described below, did not show uniform expression but instead had punctate patterns when examined under fluorescence microscopy (Supplementary Fig. 1a). This suggested that over-expression led to poor localization, and this factor was considered when selecting a transporter candidate for future engineering.

To measure metal uptake, cells were incubated in 100 \mu M metal for 4 h. Supernatant was collected and measured for remaining metal content using inductive coupled plasma (ICP), and this value was used to calculate the amount of metal removed by the cells. Parallel to each experiment a sample of wild-type (WT) and a sample with no cells were measured as controls. In addition, samples were washed in both ddH\(_2\)O and a mM EDTA buffer and measured for freed metals to account for non-specific metal binding onto the cell wall. No major non-specific metal binding was observed in either ddH\(_2\)O or EDTA wash steps (Supplementary Fig. 2). Taking these controls into consideration, enhanced uptake of zinc, copper, iron, and manganese was observed across several transporters (Fig. 1b). ZRT1,2 and CTR1,3 were highly selective for zinc and copper, respectively,
increasing metal uptake by 10-fold compared to wild-type (WT) \((p < 0.05)\). FET4 and SMF1 were less metal-specific and increased metal uptake by 3–5 fold across all four metals \((p < 0.05); \text{except for FET4 uptake of Zn compared to WT}).

A similar study was performed for arsenic and chromium. These metals are typically found in oxy-polyatomic states such as arsenate and chromate. To achieve arsenate and chromate hyperaccumulation a different set of transporters were needed. Given the molecular and steric similarity between phosphate \((\text{PO}_4^{3–})\) and arsenate \((\text{AsO}_4^{3–})\), and sulfate \((\text{SO}_4^{2–})\) and chromate \((\text{CrO}_4^{2–})\), a hypothesis was that the overexpression of sulfate and phosphate permeases would allow passage of arsenate and chromate\(^{26,29}\). Overexpression of phosphate permeases Pho84 (#P25297), 87 (#P25360), and 89 (#P38361), and sulfate permease Sul1 (#P38359) and Sul2 (#Q12325) showed increased metal uptake of arsenate and chromate, respectively (Fig. 1c; Supplementary Fig. 1a). Overall, the Pho genes increased arsenate uptake by more than 3–5 fold \((p < 0.05)\), and Sul genes increased chromate uptake by more than 5-fold \((p < 0.05)\). These observations align with plant hyperaccumulation observations that arsenate and chromate tresspass into the cell via the phosphate and sulfate assimilation pathways\(^{30,31}\).

Another common group of metal contaminants are trivalent metal ions such as aluminum and rare-earth metals. The most obvious approach would be to use a trivalent metal transport for their metal uptake; however, none exist in yeast, or generally at all. But research in a rice specie, *Oryza sativa*, uncovered a Nramp-like transporter known as Nrat1 (#Q6ZG85) which showed selective uptake of aluminum but not divalent metals\(^{32}\). Cloning and heterologously expressing Nrat1 in yeast did indeed promote selective uptake of aluminum with more than a 5-fold increase in aluminum uptake than compared to WT \((p < 0.05)\) (Fig. 1d), and no significant uptake for divalent metals such as Cu, Zn, Fe, and Mn \((p > 0.05)\) (Supplementary Fig. 3) which results align with previous published observations\(^{32}\).

**Increasing expression levels of SMF1 enhance metal uptake.**

SMF1 from the Nramp family was selected for further optimization and engineering because of its broad metal specificity (Fig. 1b), and the existing body of research on the Nramp family\(^{33–36}\). Another selection criterion was SMF1’s relatively consistent membrane-localized expression as observed under fluorescent microscopy (Supplementary Figs. 1a, 5). SMF1 was also favored because of its promiscuous activity with several metals such as manganese, iron, nickel, and cobalt\(^{27,36,37}\). Thus, SMF1 was a more appealing candidate to engineer for selective heavy metal uptake rather than converting a highly specific metal transporter which may be less malleable to change. More so, past...
work by Bozzi et al. and Ehrnströfer et al. have elucidated crystal structures of multiple Nramps and have shed light on their structure-to-function relationship with respect to metal uptake. These insights were leveraged to semi-rationally alter the metal preference of SMF1, which is shown in later results.

Enhancing metal uptake using SMF1 required increasing its expression lifetime by increasing protein yield and stability. SMF1 (denoted as S), like most nutrient transporters, is tightly regulated expression lifetime by increasing protein yield and stability. SMF1. Native yeast vacuole transporters tested were CCC1, COT1, ZRC1, and SMF3 in addition to SMF1 enhanced metal uptake. Asterisk above bar charts represent significant changes in growth rates compared to WT (p < 0.05). Constitutively expressing wheat phytochelatin synthase, TaPCS1, conferred heavy metal tolerance against cadmium. Asterisk above bar charts represent significant changes in growth rates compared to WT (p < 0.01). For all data, the mean ± s.d. of three replicates are shown. The source data underlying Fig. 2b are provided as a Data File.

Adding vacuole transporters further enhances metal uptake. Metal uptake capacity was further enhanced by expressing vacuole transporters to compartmentalized metals internalized by SMF1. Native yeast vacuole transporters tested were CCC1 (#P47818), COT1 (#P32798), ZRC1 (#P20107), and SMF3 (#Q12078) which were individually expressed in S*B strains (Supplementary Fig. 1b and 6). All tested vacuole transporters showed elevated metal uptake for copper, zinc, iron, and manganese, with CCC1 and COT1 being the most significant across all metals (p < 0.05) (Fig. 2b). These results support the role that the vacuole broadly compartmentalizes metals from the cytosol. However, without the expression of SMF1, sole expression of vacuole transporters CCC1, COT1, ZRC1, and SMF3 in WT strains had negligible impact on copper, zinc, iron, and manganese uptake (p > 0.05) (Supplementary Fig. 7). These results suggest that the largest barrier to metal uptake is from the membrane transporter, in this case SMF1, which is responsible for initial metal internalization. It is only after metal enters a cell that the vacuole transporters are rendered useful.

Phytochelatin synthase TaPCS1 enhances metal tolerance. The purpose of creating a metal hyperaccumulator becomes counterproductive if the cell dies and releases the internalized metals back into the media. Therefore, mechanisms for metal uptake...
detoxification and tolerance are needed to increase cell viability, and in theory, give cells more time to endure and uptake metals. One of the main mechanisms found in plants for metal detoxification is the production of phytochelatins, oligomers of glutathione (GSH) with cysteine and carboxyl rich moieties that chelate metals such as copper and cadmium\textsuperscript{13,15,20}. Yeast are able to produce glutathione via the GSH pathway, which naturally protects yeast from accumulation of toxic metals. However, there does not exist a phytochelatin synthase for robust metal detoxification like that in plants. Instead, yeast rely on GSH or cystein-rich and low molecular weight CUP1 metallothionein to chelate metals. However, past work has shown that metal detoxification is effective only at high copy numbers of CUP1\textsuperscript{31}, suggesting that protein production versus chemical synthesis of metal chelating compounds is less effective, possibly due to a slower rate of protein synthesis and/or abundance. Therefore, to create yeast tolerant to heavy metal environments would require a similar phytochelatin synthase mechanism. Past studies in plant hyperaccumulators have shown that a phytochelatin synthase, TaPCS1 (#0955W5), from wheat improved heavy metal tolerance in both plants and yeast\textsuperscript{42}.

Integrating TaPCS1 under constitutive expression using a GAP promoter showed cadmium tolerance beyond 100 $\mu$M, whereas WT growth rates were significantly hampered below 10 $\mu$M ($p < 0.01$) (Fig. 2c), results which support past observations\textsuperscript{42}. TaPCS1 also improved copper, manganese, zinc, and cobalt tolerance by 2–10 fold than compared to WT (Supplementary Fig. 8). The subsequent results which combine SMF1, CCC1, and TaPCS1 show that these modules can act additively to incrementally improve metal hyperaccumulation.

**Engineering a manganese and cadmium hyperaccumulator.** To mimic the characteristics of a plant hyperaccumulator, the final yeast-based system combined expression of the membrane transporter SMF1 (S, or K33,34R mutant S*), vacuole transporter CCC1 (C), metal detoxifying phytochelatin synthase TaPCS1 (T), and deletion of ubiquitin ligase BSD2 (B). All parts were integrated into the genome except for S* which was introduced on a 2$\mu$ plasmid under a GAL1 promoter. As each component was added to the system the amount of cadmium uptake increased incrementally. The effect of adding all components together (S*B*C*T) enhanced cadmium uptake by almost 16-fold than compared to WT ($p < 0.01$) (Fig. 3a). In addition, the rate of uptake increased dramatically with the combination S*B*C reaching steady-states within 2–4 h compared to 10–12 h for strains lacking an overexpressed vacuole transporter (Fig. 3b). The rate of uptake increased by almost 30-fold for S*B*C*T compared to WT ($p < 0.01$). Adding T to S*B or S*B*C did not significantly enhance metal uptake but instead stabilized metal internalization (Fig. 3a, b). After 12 h of growth in media containing 100 $\mu$M cadmium, strains without TaPCS1 began to leak back out cadmium, possibly due to cell death or activation of divergent exporters. In terms of viability, during active metal uptake in 100 $\mu$M cadmium, the expression of C slightly improved cell viability, whereas combined expression of C and T fully rescued yeast survival ($p < 0.01$) (Fig. 3c; Supplementary Fig. 9).

SMF1 and CCC1 have broad metal specificity primarily for row one transitions metals, thereby out-competing the uptake of cadmium if other transition metals such as manganese are present. To analyze the degree of manganese interference against cadmium, S*B*C*T was titrated at varying concentrations of cadmium with and without a constant background of 100 $\mu$M manganese. Metal uptake values were normalized to percent uptake with respects to the original metal concentration added, and the concentration at which metal uptake was half was termed $K_U$. The $K_U$ for cadmium with and without the presence of 100 $\mu$M went from 127 ± 12 $\mu$M to 21 ± 3.7 $\mu$M ($p < 0.01$). The $K_U$ for manganese was almost 8 times higher at 945 ± 84 $\mu$M ($p < 0.01$) (Fig. 3d). Therefore, the main mechanism of transport for SMF1 preferred manganese and the uptake of cadmium was inferred to be due to transport leakiness.

**Screening pipeline to engineer metal specific transporters.** Crystal structures and literature on Nramp structure-to-function was used to semi-rationally build libraries to create two variants of SMF1. The first variant was a more specific cadmium transporter, and the other was a strontium transporter for potential application in radioactive Sr\textsuperscript{90} remediation. The crystal structures of SMF1 homologs *D. radiodurans* (DraNramp) and *S. capitis* (ScaDMT) were used to narrow down transmembrane domains (TM) fundamental for metal recognition and transport\textsuperscript{33,34,36,38,43}. Specifically, TM regions 1, 4, and 6 in the Nramp family were identified to confer metal selectivity and movement\textsuperscript{33,38}. Without a crystal structure for SMF1, the specific TM regions had to be inferred from known structures or through multi-alignments of conserved regions. Multi-alignment of SMF1 protein sequence against a Pfam database of homologous Nramp including DraNramp and ScaDMT revealed region 76–105, 180–200, and 264–287 to represent TM1, 4, and 6, respectively, based on the highest degree of conservation when compared to TM regions in the aligned homologs (Fig. 4a; Supplementary Fig. 10).

More so, previous work in Nramp mechanistic function showed that mutation M276 in SMF1 (discovered as M230 in DraNramp) conferred metal selectivity\textsuperscript{44}. Outside crystallographic observations, it was empirically shown that mutating TM4 region G189 (discovered as G153 in DraNramp, or G185 in DMT1\textsuperscript{33,43} into an arginine exposes a calcium entryway, which was hypothesized to also transport similar group II elements like strontium (Supplementary Fig. 10).

Mutating M276C and separately G189R and M276A were performed on SMF1 prior to generating libraries for cadmium and strontium screening, respectively. Given these base mutations, error-prone PCR was done sequentially on TM1 and TM6 to generate libraries (Fig. 4b) which were then transformed into BCT strains. Creating the cadmium and strontium mutant were performed in parallel, where separate libraries were screened for cadmium or strontium uptake. During screening, libraries were subjected to either 100 $\mu$M cadmium or strontium similar to previous metal uptake experiments. Libraries were then screened based on an increase in mass as an indirect measurement for metal uptake. Mutants with higher metal content were fractionated using rate-zonal density gradient centrifugation (Fig. 4c; Supplementary Fig. 11). Rate-zonal, rather than isopycnic density gradient centrifugation was used to fractionate cells based on changes in mass, rather than equilibrium density, as previous studies have shown that yeast maintain a relatively constant density despite external influences\textsuperscript{44}. More so, our results showed the greatest segregation using rate-zonal density gradient centrifugation. Cells migrating the furthest were isolated, plated, and picked for colonies for a more focused metal assay. Cells were subjected to a competition assay with cadmium or strontium with 100 $\mu$M manganese in a 96 well format. A colorimetric assay specific to manganese was performed on the supernatant, where wells with the highest intensity (high manganese content; low manganese uptake) corresponded with mutants with low manganese preference (Fig. 4d). A select number of mutants were then chosen for quantitative metal uptake measurement using ICP, then sequenced, and later re-introduced into the
Supporting Bozzi’s et. al. work, M276 plays a critical role in metal selectivity. Changing the methionine to cysteine doubled cadmium uptake while halving manganese uptake (Fig. 5b) ($p < 0.05$). Whereas changing the methionine into alanine, and subsequently changing G189 into arginine enhanced strontium uptake while dramatically reducing uptake of manganese by almost 8-fold ($p < 0.01$) (Fig. 5b). These modifications, and each subsequent change, reduced Mn uptake while increasing uptake of Cd or Sr for mCd and mSr, respectively (Fig. 5b). It should be noted that these mutations could instead impede Mn uptake allowing increased permissiveness of Cd or Sr transport, rather than strictly increasing sensitivity for Cd or Sr; a subtle yet important distinction. However, in either case, the goal of improving Cd or Sr uptake is shown for mCd and mSr, respectively.

A titration experiment of cadmium and strontium with mCd and mSr, respectively, in the background of 100 μM manganese was performed to determine their new $K_U$. For mCd the $K_U$ for manganese dropped by 40-fold to 26.2 ± 7.6 ($p < 0.01$), whereas the $K_U$ for cadmium went from 100 ± 3.2 without manganese to 75.8 ± 10.3 in the presence of manganese, a reduction by less than 25% ($p < 0.05$) in comparison to the 5-fold decrease with the non-mutated version ($p < 0.01$) (Fig. 3d; Fig. 5c). Similarly, for mSr, the $K_U$ for manganese dropped to 17.9 ± 1.6 ($p < 0.01$) whereas the $K_U$ for strontium was 26.8 ± 5.7 and remained constant at 27.1 ± 11 in the presence of manganese.

The improved preference for cadmium and strontium uptake was more obvious when performing iterative rounds of metal uptake. When comparing uptake of cadmium or strontium in the presence of manganese for mCd and mSr against the un-mutated $S^*$, it took two rounds to fully remove manganese in the un-engineered case, while there still remained >10% manganese after 4 rounds for mCd and mSr which showed a significant reduction in manganese uptake ($p < 0.01$) (Fig. 5d). However, when measuring cadmium or strontium uptake, mCd completely removed cadmium after 3 rounds, while the un-engineered strain required 4 because of manganese uptake inhibition. For mSr, strontium incrementally decreased after each round without signs of manganese inhibition. After 4 rounds strontium levels reached below 10%, while the un-engineered strain had >80% strontium remaining ($p < 0.01$) signifying a significant change in metal preference from manganese to strontium.

**Discussion**

This work demonstrated that yeast can be engineered to hyper-accumulate metals by overexpressing and evolving native metal transporters and engineering mechanisms for metal detoxification. The main design requirements for yeast hyperaccumulation are: (1) overexpression and engineered hyperactive membrane transporter activity, (2) overexpression of vacuole transporters for metal compartmentalization, and (3) enhanced metal tolerance. Co-expression of a cell membrane transporter (SMF1) and a vacuole metal transporter (CCC1), enhanced metal uptake of manganese and cadmium by more than 10-fold, exceeding their plant hyperaccumulating threshold of 10 mg/gDW and 0.1 mg/gDW, respectively. In addition, simultaneous expression of CCC1 and plant phytochelatin synthase TaPCS1 rescued yeast survival in the presence of 100 μM cadmium. In order to improve metal selectively against the preferred manganese substrate, and more towards cadmium or strontium, information from crystallographic and empirical observations from Nramp point mutations were utilized to strategically engineer relevant SMF1 transmembrane domains. Semi-rational mutagenesis of SMF1 combined with a screening pipeline based on mass changes using rate-zonal centrifugation generated SMF1 variants with either

Creating a SMF1 transporter specific to cadmium or strontium. The SMF1 mutant with the highest cadmium specificity (denoted as mCd) contained mutations S105C, M276C, and S269T; whereas the SMF1 mutant with the most selectivity for strontium (denoted as mSr) contained mutations G189R, T266S, M276C, and G283Q (Fig. 5a). To test the contributions of each mutation, SMF1* was systematically mutated at each of the changed residues to reveal their significance and effect on SMF1 expression and function. Many of the mutations on mCd and mSr were located on TM6 rather than TM1, which supports past observations of the highly sensitive permeation region in the first alpha-helix segment of TM1 (Supplementary Fig. 12). In addition, rounds of mutations leading to mCd and mSr did not significantly change expression levels (Supplementary Fig. 13).

mutagenesis/screening pipeline (Fig. 4c). 4–5 rounds of screening were performed to generate a cadmium and strontium mutant.

**Fig. 3** Combining SMF1, CCC1, and TaPSC1 improved metal uptake capacity and tolerance. a. SMF1 (S) and its modifications ($S^*$ and ΔBSK2 as B) along with vacuole transporter CCC1 (C) and metal resistance enzyme TaPCS1 (T) incrementally enhanced cadmium uptake. Asterisk above bar charts represent significant increase in cadmium uptake when compared to WT ($p < 0.01$). b. Combinations of $S^*$, B, C, and T showed changes in uptake rate, capacity, and metal retention over 12 h of metal incubation. c. In the presence of 100 μM cadmium, the growth rate is rescued with the addition of CCC1 and furthermore with TaPCS1. Subfigure below represents the doubling time of each strain. Asterisk to the side of bar charts represent significant increase in growth rate compared to WT ($p < 0.01$). d. $S^*$BCT strain was titrated against cadmium, manganese, or cadmium in the presence of 100 μM manganese ($x$-axis). Metal uptake experiments were performed at varying concentrations from 1 μM to 1 mM, metal content analyzed using ICP, and values reported as percent uptake. $S^*$BCT showed a higher preference for manganese than cadmium, with cadmium uptake being dramatically reduced in the background presence of 100 μM manganese (light blue curve). For all data, the mean ± s.d. of three replicates are shown. The source data underlying Figs. 3a, 3b, and 3d are provided as a Source Data file.
cadmium or strontium preference and more than 10-fold reduction in manganese selectivity.

Actual application of these yeast strains in real-world settings would require another layer of technological development, such as a container or cartridge to secure yeast in a controllable unit. Fortunately, these technologies exist, such as yeast packaging, freeze-drying, and delivery which are routine technologies found in the consumer market. A potential concept would be to grow and store yeast in commercial filter-like cartridges where they can be housed in filtering units with size-exclusion cutoffs to prevent yeast leakage back into the purified waters. An additional layer of safety is to genetically modify these yeast with kill switches, or a metabolic reliance on a controlled nutrient such that removal from these containers will result in cell death.

There are yet many more handles that offer better control over metal hyperaccumulation. Expression levels of membrane metal transporters, specifically SMF1, can be enhanced by performing additional ubiquitin associated lysine mutations, deleting specific proteases such as PEP4, or integrating multiple copies into the genome with inducible or constitutive expression. Uptake could be further enhanced by trafficking metals into other organelles such as the mitochondria, ER, or Golgi which themselves

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Fig. 4 A developed high throughput screen to systematically engineer selective metal transporters. a Pfam protein database and clustering services such as ClustalΩ, TCoffee, and ESpript3 were used to align SMF1 with referenced protein crystal structure 5KTE. Through literature searches and multi-alignments, transmembrane 1 and 6 (TM1, 6) were found to be the most significant regions for mutagenesis. The alignment comparing 5KTE with SMF1 shows the TM1 and TM6 region, where yellow highlights indicate conserved regions, and red text indicate highly conserved residues (similarity score > 0.7). b Mutations cited to enhance or decrease metal transport were selectively mutated using site-directed mutagenesis. Libraries were then generated on top of these mutations through error-prone PCR. c An initial screen was performed through rate-zonal density gradient centrifugation. d, e Fractionated layers were plated, picked, and assayed for metal uptake. A competition assay of the desired metal versus the native metal (e.g., manganese) was performed calorimetrically. Wells with the least amount of native metal uptake (highest signal) were selected and quantitatively measured for metal uptake using ICP. Mutations were sequenced and reintroduced in the pipeline to generate better performing mutants.
The major benefit of using transporters for metal hyperaccumulation and environmental remediation is the control over metal selectivity. Unlike current non-specific physicochemical techniques, biological transporters engineered for metal hyperaccumulation can distinguish less abundant yet more toxic metals over background elements. Biological systems have evolved a repertoire of transporters that can be leveraged for such selective metal uptake. This work demonstrated a focused study on SMF1; however, a similar approach using the same screening pipeline can be employed to other transporters mentioned earlier. Such engineering may be limited by the lack of structure-to-function knowledge and crystal structure availability for some transporters. However, advances in nanobody-aided crystallography, NMR, and cryoTEM may help elucidate transporter crystal structures for better mechanistic understanding.

There are other areas in which yeast hyperaccumulators can have potential real-world applications. Given the customizability of yeast and methods proposed here to engineer metal selectivity, there is a possibility to design yeast strains by demand. Certain geographic areas suffer from specific metal contamination because of specific industries, for example areas in Bangladesh and India suffer from arsenic and chromium poisoning due to the textile industry. Therefore, yeast could be tailored to selectively capture and remediate arsenic and chromium from their soils. The second application is to recycle, or mine out heavy metals from solutions. Waste typically contains a mix of metals, making it extremely difficult to process and especially difficult to extract and re-capture precious metals. With this yeast-based approach it may be possible to not only remediate waste, but also to extract, concentrate, and store removed metals in yeast for mining purposes. Therefore, if a certain mixture contains X number of metals of interest, it would be possible to design X
number of strains to individually target and mine back those metals. Using yeast as a mechanism for metal removal, as well as mining and recycling can close the loop between manufacturing, use, and disposal. Therefore, rather than providing a palliative solution for the waste management crisis, yeast could be an integral tool for waste treatment processes and recycling.

**Methods**

**Yeast strain and culture.** Yeast strain W303α was obtained from the Ammon Lab at MIT. Strains of defined dropout media (SD) was made by combing 0.7 g/L nitrogen base without amino acid and ammonium sulfate (YNB) (Fischer), 5 g/L ammonium sulfate (Sigma), 0.6 g CSM-HIS-TRP-URA powder (MPBio), 20 g/L glucose (Sigma), and 10 mL/L of 100× adenosine hemisulfate stock (1 g/L) (Sigma), 100× stocks of His (5 g/L), Leu (10 g/L), Trp (10 g/L), and Ura (2 g/L) (Sigma), and in a derived media standard with similarity scores >0.7. All other amino acids are colored black. Visualized alignments identified transmembrane regions on SMFI-1 and 2, and mapped residues G153 and M230 found in 5KTE and 1M26 on SMFI, respectively. Sequence usage of the Nramp family was also visualized using WebLogo (https://weblogo.berkeley.edu/logo.cgi) onto 1M4 and 6 of SMFI to qualitatively understand the significance of mutated regions during screening.

**Transformations.** Plasmid constructions were performed in NEBx competent cells (NEB) and transformed following NEB’s protocol. Yeast transformations were performed with the Frozen-EZ Yeast Transformation Kit II (Zymo Research). The protocol was modified slightly for integrated constructs. Transformed cells were first plated onto YPAD plates and grown for 1 day. Plates were replica-plated on their respective SD drop-out and grown for an additional 1–2 days. A total of 4–8 colonies were then picked, grown overnight, and smash and glared to isolate their genomic DNA. DNA was then amplified using PCR with primers flanking the integrated area of interest and ran on a gel to verify proper integration.

**Correlating OD_{600} to culture dry weight.** Wild-type W303 were grown and varied to various culture densities ranging from 0.1–2 OD_{600} in 500 mL. Cells were pelleted and washed 3× in ddH_{2}O. 50 mL conical tubes were pre-weighted on an analytical balance with microgram resolution. Cells were transferred into these tubes, pelleted, and resuspended in 1 mL of H_{2}O. Tubes were then dipped and snap-frozen in liquid nitrogen. Tubes were then capped with a porous cloth and fitted into a lyophilization chamber (VirTis) and lyophilized for 48 h. Tubes with cells were weighed with weight of the tube subtracted to calculate cell dry weight (DW). Mass of cells per volume (y-axis) was plotted against measured OD_{600} (x-axis) giving a ratio between OD and culture dry weight per culture volume. OD to culture dry weight correlation factor was used to convert ICP results with units of µM to milligram of metal removed per gram of yeast dry weight (mg/gDW) for each strain mentioned in the results.

**Metal uptake analysis using inductive coupled plasma.** Liquid stocks of copper (II) chloride, zinc chloride, iron (II) chloride, manganese (II) chloride, cadmium nitrate, and strontium chloride (Sigma) were made at 100 mM in ddH_{2}O and filtered through a 0.22 µm filter. Colonies were streaked on SD agar plates, picked, and inoculated in SD-R media with the appropriate supplemented amino acids. Overnight were diluted 1:10 in SD-R and grown for 4 h. Cells were then pelleted and resuspended in SD-G media for induction overnight.

To prepare cells for metal uptake analysis, cells induced with SD-G were diluted to 1 OD_{600} in fresh SD-G and spiked with 100 µM metal and incubated for 4 h at 30°C. OD_{600} of metal incubation OD was measured to compare levels between strains and metals. Units were further converted to mass of metal removed per cell dry weight to help compare against literature values which report hyperaccumulation values in units of mass (mg/DW). The conversion of µM to mg/gDW required multiplying the molarity of metal removed by the molecular weight of the metal, and converting the culture OD to gram of dry weight using the radial density dry weight to weight with 0.45 being the value above.

To control for non-specific metal binding onto the cell wall, a control sample containing a wild-type W303α (WT) strain was also spiked with 100 µM metal and
processed similarly to account for non-specific uptake for in a non-expressing strain. Another sample containing no cells was also spiked with 100 μM metal to test for non-specific metal binding to the test tube and equipment. In addition, a more rigorous test for non-specific binding was performed by washing cells after metal uptake and measuring the metal content in the wash buffer. After metal uptake experiments, cells were washed once with ddH₂O to remove any residual liquid, as not all the liquid was removed for ICP analysis. Afterwards, the cells were washed once more with ddH₂O to the original volume and gently incubated for 3 min Cells were spun down, and supernatant measured for metal content. Afterwards, cells were washed another time in an EDTA buffer (10 mM Tris with 1 mM EDTA, pH 7.4) to the original volume and incubated for 3 min Cells were then pelleted, supernatant removed, and measured for metal content again.

Metal uptake titration experiments were performed following the same method but using different metal concentrations ranging from 1 μM to 100 μM or 1 mM. Metal uptake was normalized to percent uptake with respects to the original metal concentration added. The concentration at which 50% of metal was removed was termed Kᵢₑ. For interference experiments, titrations against the desired metal (cadmium or strontium) was performed in the presence of constant 100 μM manganese.

Iterative metal uptake experiments were performed by taking the supernatant of a previous metal uptake experiment, and transferring the supernatant directly into a freshly induced culture normalized to 1 OD₆₀₀ anymore. Uptake was performed for 4 h and supernatant transferred iteratively to a fresh new culture up to 4 times. At each iteration the supernatant was sampled and measured using ICP to calculate the metal uptake per round.

**Staining and microscopy.** Transporter expression was measured using immunohistochemistry. Cells were induced following the procedure mentioned above and fixed with 3.7% paraformaldehyde (EMC) at 0.5 OD₆₀₀ for 30 min at room temperature. Cells were then pelleted, washed, and resuspended in 1.2 M sorbitol buffer (Sigma) before being placed in the same buffer with 1:100 dilution of 100T Zymolyase (Zymo) and incubated at 30 °C for 30 min to 1 h. Cells were pelleted and washed 3x in PBS + 1% BSA before settling on poly-l-lysine treated 8 well chamber slides (Lab-Tek). Cells were gently permeabilized with 0.1% TWEEN 20 (Sigma) in PBS + 1% BSA on ice for 5 min. Cells were then stained with the appropriate primary antibody and dyed with a live-dead fluorescent indicator (Thermo). A positive control of freshly grown cells, and a live-dead assay was also performed to analyze cell viability by calculating the ratio of live to dead cell populations, respectively. Counts within those gates were used to calculate ratio of live cells after metal uptake. Cells were analyzed under the FITC and PE channels of an LSR II flow cytometer.

**Manganese assay.** The manganese colorimetric detection Hach kit was modified to fit a 96-well format. Fifty microliter of sample was added to 50 μL of 2x ascorbic acid provided by the kit. Then 5 μL of the cyanide and PANI reagent were used to detect manganese given a colorimetric change from yellow to red. Wells were measured at 560 nm. Cyanide was disposed of using guidelines approved by MIT EH&S.

**Screening transporter libraries.** Percoll (Sigma) buffered with 1.5 M NaCl was used to make density gradients. A Pharmacia LKB Pump P-1 peristaltic pump joined to a gradient maker (GE) was used to make Percoll gradients. Gradinets were formed in Greiner 16 x 100 mm round bottom polystyrene tubes (Sigma) which were first hydrophobic coated with SigmaCoat (Sigma). A purple dye was used as a control to visually inspect consistency of gradient formation per batch. Libraries were transformed into yeast and plated. Single colonies were pooled together using a scraper (Corning) into 10 mL of SD-R with the appropriate amino acids. Cells were grown for 12 h before being diluted into 50 mL of SD-R for 4 h. Cells were then pelleted and resuspended in SD-G with the appropriate amino acids for induction overnight. Induced culture was then diluted to 1 OD₆₀₀ into multiple 10 mL SD-G media with spiked 100 μM of cadmium or strontium. Cultures were grown for 4 h before washing and resuspending in 150 mM NaCl. Cells, percoll gradient, and an Apperdorff 5804-R swinging bucket centrifuge were centrifuged at 16 °C before spinning. Settings for acceleration and braking were set to 0. Cells were gently layered onto the gradient and spun in increments of 5 min at 100 x g. A total of 3–4 spins were sufficient to observe segregation of cells which signified a fractionation of heavier cells due to metal uptake. Approximately a centimeter below the least visible band was collected and spun down at 1500 x g for 3 min before resuspending in SD with the appropriate amino acids. Cells were rescued for 1.5 h before plating. Collected cells were plated onto 2–3 plates giving approximately 10–100 colonies each.

After plating roughly 10–50 colonies were picked in 100 μL SD-R cultures in a 96-well format and induced following the same protocol as before. Cells were grown for 1 OD₆₀₀ and spiked with 100 μM cadmium or strontium with the addition of 100 μM manganese and shaken for 4 h. Plates were spun down at 900 x g for 3 min and the supernatant was diluted 1:10 in ddH₂O and assayed using the modified manganese Hach detection kit described above. The top 4-6 wells with the highest readings (most manganese remaining) were selected and plated again. Selected colonies were then subjected to a more thorough metal uptake ICP experiment and sequenced before re-introduction into the screening pipeline.

**Mathematical analysis and plotting.** Raw data were collected and stored as csv or excel file formats. Data were imported and analyzed with python using modules such as numpy, pandas, and scipy. Plots were graphed using matplotlib.

**Statistical analysis.** Statistical parameters including the definitions and values of n, SDs, and/or SEs are reported in the figures and corresponding figure legends. When reporting significance, a two-tailed unpaired t-test was performed for all calculated p-values. The significance threshold was set to p < 0.05 for all experiments, as specified in the text.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data supporting the findings of this work are available within the paper and its Supplementary Information file. A reporting summary for this Article is available as a Supplementary Information file. The datasets generated and analyzed during the current study are available from the corresponding author upon request. The source data underlying Figs. 1b, 2b, 3a, b, d, and 5c, as well as Supplementary Figs. 2, 3, 7 and 9 are provided as a Source Data file.

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