Characterization of Met25 as a Color Associated Genetic Market in *Yarrowia lipolytica*

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Abstract

Yarrowia lipolytica offers an ideal host for biosynthesis of high value natural products and oleochemicals through metabolic engineering despite being restricted to a limited number of selective markers, and counter-selection achieved primarily with URA3. In this work, we investigate MET25, a locus of sulfide housekeeping within the cell, to be exploited as a marker. Divalent lead supplemented in media induces lead sulfide (PbS) aggregation in MET25-deleted cells such that deficient cells grow brown/black, and cells with functional copies of MET25 grow white. It is hypothesized that the sulfide buildup from MET25 deficiency can also be exploited to neutralize and detoxify methylmercury by formation of mercuric sulfide (HgS) and ethanol, which confers toxic resistance to methyl-mercury. A plasmid-based expression system of the CRISPR-Cas12 nuclease coupled with crRNA targeting MET25, was also employed as a gene disruption technique to orthogonally demonstrate MET25 as a locus responsible for this phenotypic change and to test this locus as a target for our gene disruption platform. Kinetic growth curves of wild type and MET25-deficient cells were obtained under varying concentrations of methylmercury. Cellular toxicity to methyl mercury was calculated from the Hill equation. Furthermore, MET25 did not induce strict auxotrophic requirements for methionine in *Y. lipolytica.* Plasmid and chromosomal-based complementation of MET25 deficient cells on a double layer agar plate with gradients of lead and methionine demonstrates delayed phenotype restoration, indicating post-transcriptional regulation of methionine in this yeast.

Keywords: methionine biosynthesis, *Yarrowia lipolytica*, genetic marker, color associated screening, counter selection, CRISPR-Cas12/cpf1.

Running title: Sulfur house-keeping metabolism in oleaginous yeast

Introduction

With the limited number of auxotrophic markers in the oleaginous yeast *Yarrowia lipolytica*, establishing an additional counter selectable marker has potential to add significant value and versatility to the genetic toolbox with regards to engineering this host organism [1]. *URA3* is the conventional selection marker used in a few yeast species including *Y. lipolytica* because it offers auxotrophic transformant screening as well as counterselection potential for marker removal (*via* 5'-FOA resistance) [2, 3]. Great advancements in genome editing have been made in this host recently despite largely being restricted by this single genetic marker [2, 4-8]. Counter selectivity is essential for performing iterative chromosomal gene integrations and ensuring that random integration of the selectable marker from the first round of genetic manipulations does not lead to false positives upon use of that marker in subsequent transformations[2]. The *LEU2* marker requires similar synthetically defined media, and an auxotrophic host strain[9, 10], but without any known methods offering counter selection. Hygromycin and the *hph* dominant marker give the advantages of antibiotic resistance selection[11, 12], largely decoupling cell death and fitness from essential nutrients, but includes disadvantages like dose-independent spontaneous resistance and still no counter selectivity [13].

The *MET25* is a gene found in *Y. lipolytica*, homologous to *MET15* found in *S. cerevisiae*, and various pathological species of *Candida*. *MET25* in *Y. lipolytica* encodes for O-acetyl homoserine sulfhydrylase (EC 2.5.1.49) with a length of 425 amino acids. This enzyme is found ubiquitously in various organisms[14, 15], and is reported to be responsible for catalyzing over 40 reactions involved in sulfur metabolism. Some of these reactions are listed in Table 1, almost all of which are involved in metabolism of amino acids containing sulfur. This enzyme will catalyze an acetyl transfer reaction between methanethiol and O-acetyl-L-homoserine to produce methionine and acetate, reaction 1. Reactions 2, 3, and 4 indicate the use of hydrogen sulfide (H_2S) as a substrate in conjunction with other carbon backbone substrates. This demonstrates significant trans-activity with respect to various small carbon sources and the fixation/sequestration of hydrogen sulfide.

The naming of this protein is understandably a point of confusion, as this locus has been

targeted by many other groups, in various hosts. *MET25*, *MET15*, and *MET17* are essentially synonymous in KEGG databases or Addgene catalouges. Since the realization of *MET15*, as named in some sources, as a phenotypic locus also associated with methyl-mercury resistance [16, 17], a color associated counter selectable marker was discovered in *S. cerevisiae* [18], and this genetic tool has become a standard auxotrophic marker in *S. cerevisiae* strain BY4741[19, 20]. Similarly, *MET15* has been utilized in *C. albicans* as a positive/negative color associated selection marker [21]. *MET25* as a name seemingly came along when it is found that the promoter for O-acetyl homoserine sulfhydrylase in *S. cerevisiae* was highly tunable to methionine concentration [22], and another group studying sulfur amino acid biochemistry in *S. cerevisiae* at the time also published a pathway labeling our gene of interest as *MET25*[23]. The *MET25* promoter is sold commercially due to the tight tunability to methionine concentration. YLR303W is the most consistent point in the literature for naming this gene and we will refer to it as *MET25*.

The recent development of genome-editing tools have made *Y. lipolytica* an ideal host for various applications, ranging from biofuel production[24-26], natural product biosynthesis [27-29] and commodity chemical manufacturing[30, 31]. To further expand the genetic toolbox and understand the sulfur metabolism, we hypothesized that *MET25* could be utilized as a counter selectable color-associated genetic marker in *Y. lipolytica*. In this report, we used both the homologous recombination and the CRISPR-Cas12/cpf1 nuclease to disrupt *MET25*. We characterized the phenotype of *MET25*-deficient cell under the colorogenic media (soluble lead acetate) or counter-selectable media (methyl mercury). With plasmid-based or chromosome-based complementation of *MET25*, we also validated whether the phenotype could be restored. Based on a double-layer slanted agar with gradients of lead (Pb^{2+}) and methionine, we inferred the regulatory mechanism underlying methionine biosynthesis. The development of *MET25* may further expand our ability to enable *Y. lipolytica* as an oleaginous yeast for various biosensing, bioremediation, bioproduction and biomedical applications.

Results

Chromosomal disruption of MET25 *via* homologous recombination

We first attempted to disrupt *MET25* via the conventional homologous recombination methods, a *URA3* disruption cassette. After transformation onto 5'-FOA containing plates of minimal media, a black phenotype was observed on 37.5% (3/8) colonies picked from that transformation and spotted onto MLA. (Supplementary Figure 1). This did not guarantee a true homogeneous population of *ΔMET25* mutants due to the possibility of mixed colonies from the CSM-Ura plate. This non-homogeneous population was confirmed by serial dilution and spreading on another MLA (modified lead agar) plate in which one can observe the phenotypic separation of white and black colonies (Fig. 1A). This colony isolation was repeated (Supplementary Figure 2.A) until a true homogenous population of black colonies was observed, (Supplementary Figure 2.B)

Results regarding methionine auxotrophic requirements vary from reports in other yeasts. Knocking out the *MET25* locus in *Y. lipolytica* did not produce a strict methionine auxotroph as *MET15* does in *S. cerevisiae* [18]. Similarly, knockout of the appropriate homologue in *C. albicans* did not create an auxotroph [21]. In order to test whether *MET25* is a strict methionine auxotrophic marker, colonies derived from Po1f, Po1f Δ*Met25*, and Po1fΔ*Met25* with pYLXP'-*Met25* were spotted on various media to confirm *MET25* deficiency and *MET25* restoration (Figure 2, and Supplementary Figure 3-6). The double dropout media, CSM-Leu-Ura plates, were used as controls for the three genetic variants to ensure the correct genotype (supplementary figure 5). CSM-Met plates demonstrated to have little to no effective difference, indicating the existence of a methionine rescue pathway or contributions from media when spotting the plates. To rule out the pre-culture media effect, the cells were washed with PBS buffer and plated again onto CSM-Met (supplementary figure 3). We observed less growth in *ΔMET25* mutants, indicating clear cellular burden imposed on *ΔMET25* cells, although it did not induce a strict requirement for methionine. Cystathionine beta-lyase, *METC*, (YALI0D00605g) was hypothesized as the potential rescue pathway. The hypothesis was by inhibiting the critical intermediate reaction between converting cystathionine to methionine, a methionine auxotrophic

strain may be observed. The double knockout Po1fΔ*Met25+ylUra3*Δ*MetC*, also grew (data not shown) on methionine deficient media (CSM-Met), albeit with a diminished growth rate. We concluded that cystathionine beta-lyase does not appear to be a critical enzyme in the methionine rescue pathway responsible for conferring growth to our host without methionine.

Genome-editing of MET25 *via* CRISPR-cas12/cpf1 nuclease

We also sought to disrupt the *MET25 via* CRISPR-cas12/cpf1. After co-transformation of Po1f with the dual expression plasmid pYLXP'-*AsCpf1-AsCrRNA-Met25*, colonies grew on CSM-Leu plates as typical. Colonies from those plates were then rescreened on MLA and grew with a white colony formed in one day, and 72 hours for visible black sectoring to be observed in 1/16 samples. By the end of 7 days 6 out of 16 sample had black sectors and after 14 days 11/16 had visible sectors (Figure 3). This indicates the genome-editing of *MET25* with CRISPR-Cpf1 depends on prolonged genome-targeting and cutting, as makes sense since the Cpf1 protein and associated RNA need time to be synthesized, folded, and located properly in vivo for their individual roles to be completed together. The MET25 locus provides an easy target for testing gene disruption efficiency with a phenotypic indication. MET2(YALI0E00836g), and *MET6(*YALI0E12683g) were all independently targeted with the same plasmid based Cpf1 gene disruption platform with success in achieving phenotypic selection[32].

Delayed phenotype restoration of MET25 deficiency indicates orthogonal feedback regulation of methionine biosynthesis in *Y. lipolytica*

We next investigated whether we could restore the white colony phenotype by complementing *MET25* deficiency using either plasmid or chromosomal-based expression of *Met25*. Use of *MET25* alone in rich MLA media has proven difficult due to the lack of counter-selection pressure for negative transformants: almost all populations of transformants were black on the MLA plate, indicating either low transformation efficiency or negative transformants. To overcome this limitation, we next pursued a 2-step screening: we first screened the *LEU2* marker on CSM-leu plate to ensure expression of *MET25*, then the isolated colonies were replicated to MLA plate to validate phenotype restoration.

Episomal or chromosomal expression of *MET25* in *ΔMET25* cells proved difficult to restore the white colony phenotype, but the results of continued interest alluded to interesting regulation of methionine. Upon complementation with this *MET25* deficient host, the white phenotype was not observed initially (Fig. 1B). The cells grow in CSM-Leu, indicating the retention of the *LEU2* marker from the plasmid. Re-streaking the colonies isolated from CSM-Leu also grew black on MLA, indicating no functional complementation of *MET25* from the plasmid or chromosomal-based *MET25* expression cassette, despite functional *LEU2* expression. After leaving the black transformant colonies in the incubator past 7 days, a white ring appeared radially surrounding a black core (Fig. 1B), as more generations grew. Colonies isolated from the white ring were re-streaked on MLA plate, demonstrating the similar patterns: a black core was surrounded by a white ring. MLA is rich media, and CSM-Leu/CSM-Met cannot stably contain the divalent lead without salting out so we cannot perform this phenotypic assay in minimal media. We are confident in our genetic knockout from sequencing and extensive colony isolation and hypothesize that media contributions of methionine were causing the effect. Results from the first CSM-Met assays (Supplementary Figure 3), preliminarily indicated an extremely sensitive feedback mechanism to inhibit methionine synthesis by methionine.

This was further validated with the double-layer, slanted agar plate assay with a gradient concentration of lead (Pb^{2+}) and methionine (Figure 4). The increasing size of the white ring was accompanied with a decreasing methionine concentration (Fig. 4), in both the plasmid and the chromosome-based complementation of the *MET25* gene. This assay confirmed that methionine was negatively autoregulating the functional expression of the *MET25* gene. For example, the critical outer diameter of the white ring was observed to be inversely proportional to the local methionine concentration (Fig. 4). In order to ensure it was methionine diffusion, a slanted agar plate was poured with MLA on bottom and CSM-Met on top and no coloration was observed (Fig. 4), indicating that Pb^{2+} diffusion was not causing the fitness change of the cell.

The white ring of the *MET25* complementation experiment suggests the methionine existing in the MLA media may produce feedback to inhibit the translation of *MET25* mRNA

transcripts in *Y. lipolytica*, since it was orthogonally expressed via the TEF2 and XPR2 promoter in our plasmid. The delayed phenotypic restoration in radially growing colonies indicated that there was sufficient amount of methionine inhibiting the expression of *MET25* in early growth stage, as a result, expression of *MET25* was not required, sulfur was not utilized, and the colonies remain black. At a later time when methionine was limited or depleted, expression of *MET25* was required, utilizing sulfur, and the new cells grew with their original white phenotype. It has been seen that there are multiple levels of regulation for MET25 and methionine synthesizing pathways, including activating sequences of promoter regions, and post transcriptional interactions between methionine and the 5' region of the mRNA transcript[33]. In our case, the timing of the phenotypic shift is affected by methionine concentrations when expressed under an orthogonal promoter (TEF2) and terminator (XPR2), whether plasmid or chromosomally expressed. Our results allude to allosteric effects of methionine and Met25 the enzyme, co-repressor effects between methionine and an orthoganlly expressed transcriptional factor or post transcriptional activity between methionine and the MET25 transcript.

MET25 deficient yeast as a whole cell senor to detect lead in potable water

Heavy metals in water have been linked with many diseases and have long been associated with neurodegenerative conditions in both kids and aged population. Since *MET25* deficiency leads to black pigmentation and form PbS precipitates, *ΔMET25* cells may be used as a whole-cell sensor to detect lead in potable waters. In order to gauge the applicability of this strain for microbially-based lead sensing purposes, the *ΔMET25* mutants were cultivated on MLA media containing 1000, 100, 10, and 0 mg/L (1000ppm), of soluble lead (II) nitrate, (Figure. 5). Yeast colonies and surrounding media were darkened in 100 ppm lead, by 24 hours, and significantly darker by 48 and 72 hours. Colonies gown on 10 ppm lead(II) MLA plates were visually darker after 72 hour's cultivation (Fig. 5). These results indicate a high level of sensitivity could be achieved by a simple, microbial-based lead bioassay, reaching the detection limits of 10ppm (Fig. 5). Further genetic engineering may be needed to tune the sensitivity and dynamic range of this whole cell microbial probe even further. If the local sulfide concentration can be increased or sulfate-related transporter genes could be manipulated this organism could

provide a scalable and effective, low cost, non-electric whole microbe sensor for heavy metals.

Growth rates and counter-selection test toward methylmercury

We next attempted to determine the strength of the counter-selectivity toward methyl mercury in the *MET25* deficient strain. This specific growth rates for Po1f and Po1fΔ*Met25* were obtained by the slope of the linear regression line of OD data versus time plotted on a logarithmic scale (equation No. 1). Then the specific growth rates were plotted against the level of methyl mercury to quantify the dose response relationship. Analyzing these specific growth rates in YPD at varying concentrations of methyl mercury in 96-well plates demonstrates a well fit with a Hill-type equation, governing the sigmoidal relationship between specific growth rate and methyl mercury concentration (Figure 6) Table 2 contains the complete list of parameters values. The half inhibitory constants for the Po1f and Po1fΔ*Met25* against methyl mercury were estimated at 0.746 μM and 1.383 μM respectively. As expected, with the deletion of the *MET25* gene, the mutant cells become more resistant to methyl mercury although by such a small margin, the practical applications (i.e. genetic selection with a <0.5 μM window of counter selectivity) are difficult to achieve.

Discussion

Disruption of *MET25* locus in *Y. lipolytica* via homologous recombination, or through targeted indel knockouts, certainly induces the formation of brown/black colony in the presence of lead (II). The Δ*met25* mutant generated visibly darker coloration to the naked eye on media containing as low as 10 ppm lead. These findings alone indicate a potential for biochemical engineers to develop a low cost, easy to use, microbial lead sensor for point of care applications in regions with polluted water. Further genetic engineering of microbial sulfur metabolism could increase lead detection sensitivity by increasing intracellular sulfide availability through limiting other metabolic steps that consume sulfide. The formation of lead (II) sulfide alludes to other possible sulfide and heavy metal chemistry, including the formation of color distinct cadmium sulfide or cadmium selenide.

CRISPR-Cpf1/Cas12-mediated genome editing targeting the *MET25*, *MET2*, or *MET6* locus was able to successfully induce black colony sectoring in Po1f, indicative of successful

indel mutation or gene knockout[32]. These loci *MET25*,*MET2*, and *MET6* all provide similar behavior to target any of those genes for knockout, and a subsequent phenotypic screening. Knocking out any one of these *MET* genes individually does not induce an auxotrophic requirement for methionine, indicating that methionine can be biosynthesized via alternative route. Cystathionine β -lyase, EC.4.4.1.13, or *METC*, was knocked out, along with *MET25*, creating a double mutant which still was able to synthesize methionine to sustain growth. There is a methionine-adenine salvage cycle reported in plants and bacteria[34], and we identified homologous proteins for the methionine salvage pathway in *Y. lipolytica*, pointing future genetic engineering targets there to engineer a strict methionine auxotrophic strain. With the ubiquitous cellular requirement for methionine, considering it is also the start codon, it is likely advantageous for being redundant in housekeeping of enzymes responsible for methionine biosynthesis.

The chromosomal deletion of *MET25* induced resistance to the toxic chemical methyl-mercury and a Hill type relationship was demonstrated between the specific growth rate and the methyl-mercury concentration. The difference in half inhibitory constant (IC_{50}) between the wildtype and mutant were about 0.35 μM, and this represents a challenge in exploiting this small window for counter selection. Since growth curves were in liquid media, those numbers were not the most help in determining a useful screening concentration of methyl-mercury on agar plates, and plate screening proved continuously difficult. Counter selection and phenotypic screening cannot be combined easily as the same intracellular sulfide affording a slight resistance to methyl mercury, is the same sulfide covalently reacting with lead to form a PbS black precipitate. If sulfide is utilized for color indication, it cannot also save the cells by neutralizing methyl mercury. Phenotypic screening and dropout media also could not be done together due to lead salts forming with nutrients in minimal media.

Most interestingly, even when *MET25* is expressed orthogonally via an independent promoter and terminator set (p*TEF2* and XPR2), there is still negative autoregulation of *MET25* activity due to local methionine availability. Further work should investigate differential gene expression under these conditions, and quest for the transcriptional factors (TFs) involved in this

regulation, as well as perform calorimetric and energetic binding assay between the TFs, methionine, the *MET25* template DNA, and the *MET25* mRNA transcript. This may help us uncover a potentially novel feedback regulation mechanism in methionine biosynthesis apart from the commercialized *MET25* promoter. Considering the phylogenic age of the amino acid located at each and every start codon, there may be a robust and ubiquitous method of regulation like the tryptophan attenuation loop, or this could be a complex interaction network of transcription factors as is often not completely understood in eukaryotic signaling networks. Further investigation into the mechanism of feedback for methionine and *MET25* should be pursued for mechanistic discovery and also to standardize the phenotypic screening with the sulfur-housekeeping marker in *Y. lipolytica*.

Conclusions

The *MET25* marker in *Y. lipolytica* can offer phenotypic screening of transformants quite easily on rich media supplemented with lead(II)acetate. Positive screening with *MET25* and solely lead has proven difficult as there is no negative selection pressure to limit growth of negative transformants and a methionine auxotrophic strain was not observed either. To overcome this limitation, counterselection is narrowly achieved via growth on rich media containing methyl-mercury. Methyl-mercury is toxic, and could be detoxified by sulfide buildup in the *ΔMET25* cells, due to the formation of mercuric sulfide and ethanol conferring toxic resistance to methyl mercury. The counter-selection window, at under 0.5 micromolar in liquid media, makes this technique difficult to practically achieve. Both positive/negative screening, and counter selection can be done in rich media, although not in a single assay/plate due to synergistic effects of sulfide on both the mechanism of toxic resistance and the mechanism of phenotypic distinction.

MET25 in our system was overexpressed via the *TEF2* promoter and XPR2 terminator, but still, methionine based autoregulation of this gene, observed in the slanted plate agar test, increased difficulty in consistent results with this strain. Counter selection was barely achieved and the narrow window of counter selectivity makes this technique difficult. Combined with the potential health hazards associated with mercury reagents and bioassays which use them,

methyl-mercury selection is less than ideal. Further genetic engineering should work to build a methionine auxotroph, which would alleviate the necessity for methyl mercury counter selection, and should focus on increasing the rate and magnitude of this sulfide buildup to further leverage phenotypic and genetic selection traits. Utilizing these methods in rich media has potential to increase the rate at which transformants grow and are screened, which may significantly decrease the time spent on engineering this host. This selectable marker also facilitates better understanding of cellular regulation because of the visual indications of genetic events indicated by sectoring or delayed phenotype expression.

Materials and methods

Plasmids, strains and media

Y. lipolytica Po1f (ATCC MYA-2613, MATA ura3-302 leu2-270 xpr2-322 axp2-deltaNU49 XPR2:SUC2) was used as the host strain. *Escherichia coli* NEB5α was used for plasmid construction and proliferation. The YaliBrick plasmid pYLXP' was used as the backbone to construct other plasmids [1]. LB broth or agar plates containing ampicillin (100 mg/L) was routinely used for *E. coli* cultivation. YPD media consisting of 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose and complete synthetic media (CSM) omitting proper amino acids were used for yeast cultivation and transformation. MLA plates containing 3 g/L peptone, 5 g/L yeast extract, 0.2 g/L ammonium sulfate, 40 g/L glucose, 1 g/L lead nitrate and 20 g/L agar were used for visual selection of *met25* mutants. Lead nitrate was filter sterilized and added to autoclaved mixture of other components after cool down.

Met25 Disruption via Homologous Recombination

All primers used in this work were listed in supplementary table 1. To construct a cassette for the deletion of *MET25*, primers met25upfw and met25uprv were used to PCR amplify an 800 bp fragment immediately upstream from the start codon of *MET25* using genomic Po1f DNA as template. This fragment was size verified via gel electrophoresis and purified using ZYMO Clean and Concentrator kits. Another 800 bp fragment immediately downstream from the stop codon was obtained using primers met25dwfw and met25dwrv. The gene ylUra3 had

previously been functionally cloned in our lab, into the plasmid pYLXP', to create pYLXP'-*ylUra3*. That plasmid was linearized with *Sal*I and gel purified. The downstream 800 bp fragment was cloned into the *Sal*I digested vector backbone to yield pYLXP'-*ylUra3*-Met25DW, via Gibson assembly, transformed into *E. coli* and screened on LB agar plates.

Colonies were verified via colony PCR using xpr2_fw and met25dwrv. Positive colonies were inoculated into LB media containing ampicillin for overnight culture. Plasmid was purified using ZYMO Miniprep kits and sanger sequenced. The upstream 800 np fragment was cloned into pYLXP'-*ylUra3*-Met25DW digested with *Cla*I to yield pYLXP'-*ylUra3*-Met25. The primers tef-rv and met25upfw were used for colony PCR to screen colonies for a 900 bp fragment. The sequencing-verified pYLXP'-*ylUra3-Met25* was used to PCR amplify a deletion cassette of *MET25* using primers met25cassfw and met25cassrv.

The *MET25* knockout cassette was transformed into wild type Po1f strain using hydroxyurea-based protocol to enhance homologous recombination[35],[4]. Transformants were plated onto CSM-Ura plates. These preliminarily positive transformants were diluted into 10 µL sterile water and then used for selective media assays. These transformants were screened on MLA plates (Supplementary figure 1) and colonies which turned dark in color were picked to inoculate in CSM-Ura liquid media. That liquid culture was diluted and streaked on MLA to perform colony isolation. A single black colony was picked and inoculated into CSM-Ura liquid media, incubated 24 hrs, diluted, and re-plated to ensure that a true homogenous population of *ΔMET25* mutants was obtained. (Supplementary figure 2)

Plasmid and Chromosomal Complementation of Met25

MET25 was PCR amplified out of genomic Po1f DNA using the primers met25fw and met25rv and cloned into vector pYLXP' to yield pYLXP'-Met25. This plasmid was transformed into *ΔMET25* cells and plated onto CSM-Leu plates. Positive transformant were plated onto various selective medias (Supplementary Figures 3 to 6) for comparison of growth behavior and *MET25* expression in the wildtype Po1f, mutant Po1f+*ylUra3-Met25*, and mutant containing

plasmid Po1f+*ylUra3-Met25* and pYLXP'-*Met25*. A graphic summarizing these plates can be observed in figure 2.

Growth assay in CSM-Met selective media was performed in order to test if *MET25* knockout could confer auxotrophy for methionine. Results were negative, as po1f-*ylUra3-ΔMET25* grew on CSM-Met plates when CSM-Ura liquid culture was used. The experiment was repeated to see if methionine in the liquid media was enabling growth. In this case cell cultures of each sample were grown for 2 days in appropriate selective media and centrifuged at 1800xg for 10 minutes to pellet cells. The supernatant was discarded, the pellet was resuspended in PBS and this entire wash was repeated to ensure no nutrient from the media is transferred with the cells that inoculate the selective media plates. These two plates are observed in supplemental figure 3.

To construct a strain with chromosomally integrated expression cassette of *MET25*, pYLXP'-*Met25* was linearized with NotI, a restriction site flanked by a chromosomal landing pad of complementary bases[1]. The linearized plasmid was transformed into mutant cells and plated onto CSM-Leu plates. Colonies were inoculated into YPD liquid media for 72 hours and spread onto CSM-Leu plates. Single colonies from this plate were taken as chromosomal integrations of the plasmid.

Met25 Disruption via CRISPR/Cpf1 Mediated Indel Mutation

In order to gauge the applicability of CRISPR/Cpf1 mediated, transient gene disruption of a phenotypic locus, and to orthogonally demonstrate the responsibility of the *MET25* gene in the lead(II) sulfide producing cells, a single plasmid was created containing a functional copy of AsCpf1 and AsCrRNA_Met25. AsCrRNA_Met25 is the guide RNA created analogous to the gRNA of *CAN1* design[1]. The construction of CRISPR-Cas12 plasmids used to knockout *MET25*, *MET2*, and *MET6* can be found in literature[32, 36]. pYLXP'-*AsCpf1-AsCrRNA-Met25* was transformed into Po1f and plated onto CSM-Leu, incubated for 72 hours, and colonies were screened on MLA plates by picking single colonies into 10 μL sterile water, and spotting 3 μL on CSM-Leu, and 3 μL on MLA. These two plates were incubated for 48-72 hours (Supplementary Figure 4).

MetC, and Met25 Double Knockout

A gene disruption cassette utilizing *ylUra3* marker was created and employed, using identical techniques as described in section 2.1, this time with 800 bp homologous arms designed to initiate homologous recombination in the *METC* (YALI0D00605g) locus. Colony PCR verification was performed using MetCupchk and tef-rv, as well as MetCdwnchk and xpr2-fw, for a 900 and 1000 bp fragment indicating successful integration, respectively. Positive colonies from colony PCR verification are now grown in CSM-Ura and designated as Po1f+*ylUra3-MetC*.

pYLXP'-*AsCpf1-AsCrRNA-Met25* was then transformed into Po1f+*ylUra3-MetC*. Transformants were grown on CSM-Leu for 72 hrs. Colonies were then picked, resuspended in 10 µL sterile water, and plated onto MLA plates. Once black sectoring was observed, this colony was chosen, diluted and spread onto MLA to isolate a single, black colony. This took three rounds of colony resuspension, dilution, plating, and isolation, before a homogeneous population was observed. This transformant was designated Po1fΔ*Met25+ylUra3-MetC*. This strain was assayed for an auxotrophic methionine requirement on CSM-Met plates.

Selective Media and Differential Growth Test

All selective media agar plates use 2% agar. Dropout media CSM-Leu and CSM-Ura plates were used to selectively screen positive transformants. CSM-Met and CSM-Leu-Ura were employed to observe burden associated with different autotrophies, and to verify plasmid holding mutants respectively. Modified lead agar, MLA, plates were made with 1 g/L lead(II) nitrate[37], [18] YPD plates containing 4µM and 8µM methylmercury were used to investigate counter-selectivity. In order to establish a gradient of nutrients, agar plates were partially filled with CSM-Met, and the plates were tilted and allowed to cool such that the gel set diagonally. The plate was then poured the rest of the way with MLA media, ensuring to cover the minimal media entirely, and allowed to set again. This was repeated vice versa with MLA media on bottom and CSM-Met on top too. The result of these media assays with an established nutrient gradient are found in Figure 4

In order to more quantitatively observe the utility of methylmercury as a counter-selection

agent, specifically in Y. lipolytica, growth rates were measured in liquid media at varying concentrations of the methyl mercury. Po1f and Po1f+ylUra3-Met25 were inoculated in YPD and cultured for 24 hours. OD600 was normalized at 0.15 in the wells, and measurements were taken on a 96-well microwell-plate reader, during incubation at 30 degrees C with full shaking. OD was measured every 10 minutes for 8 hours. These readings were fit linearly on a logarithmic scale and the slope recorded as the specific growth rate. The specific growth rate was normalized with the maximum value to determine the relative growth rate labeled in percent of the maximum specific growth rate. These specific growth rates were plotted at various concentrations to visually establish a relationship for Po1f and Po1fΔMet25. Specific growth rates can be estimated by equation No. 1; and the inhibitory constant could be determined by the following Hill type equation (equation No. 2).

$$\mu = \frac{d\ln(OD)}{dt} \qquad \text{Equation.1}$$

$$\mu = A_1 + \frac{(A_2 - A_1) * X^n}{K^n + X^n} \qquad \text{Equation.2}$$

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Author contributions

PX conceived and designed the topic. HE and ZY performed genetic engineering. HE wrote the manuscript. PX and ZY revised the manuscript.

Conflicts of interests

There are no conflicts of interest to report in this work.

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Tables

Table 1. Short list of possible reactions catalyzed by O-Acetylhomoserine aminocarboxypropyltransferase

Reaction	Substrate	Products
1	O-Acetyl-L- homoserine + Methanethiol	L-Methionine + Acetate
2	O-Acetyl-L- homoserine + H_2S	L-homocysteine + Acetic Acid
3	O-Acetyl-L-Serine + S^{2-}	L-Cysteine + Acetic Acid
4	O-Succinyl-L-Serine + H_2S	L-Cysteine + Succinic Aceid

Table 2 Regression Values For Hill Type Equation

Hill Equation Regression Values		
	Po1f	Po1fΔMET25
R^2	0.998	0.995
A1	1.00	0.999
A2	0.096	-0.10
k	0.757	1.45
n	1.35	1.23

Figures

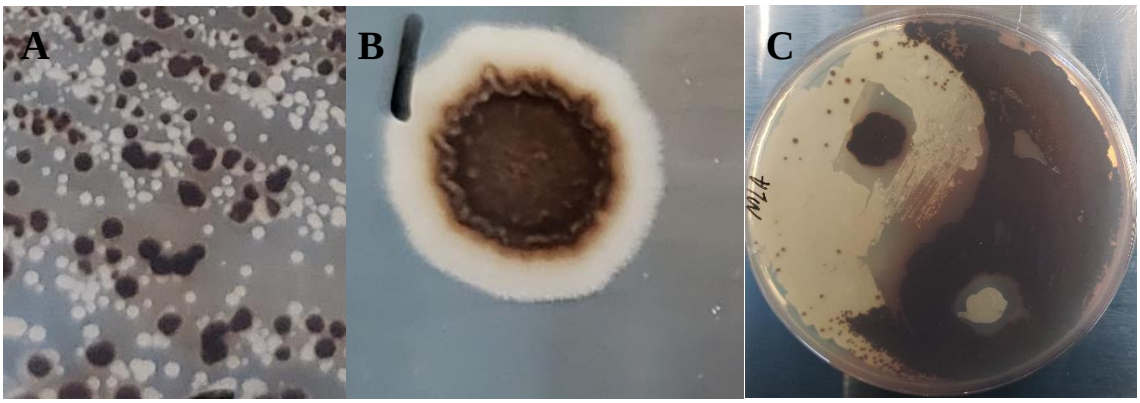

Fig. 1 Phenotypic separation of white and black colonies after MET25 deletion (A). Plasmid-complementation of MET25 in the MET25-deficient cell leads to the formation of a radially distributed white ring (B). Yin-Yang art (C) of wild type (white colony) and MET25 deficient cells (black colony) on MLA plate.

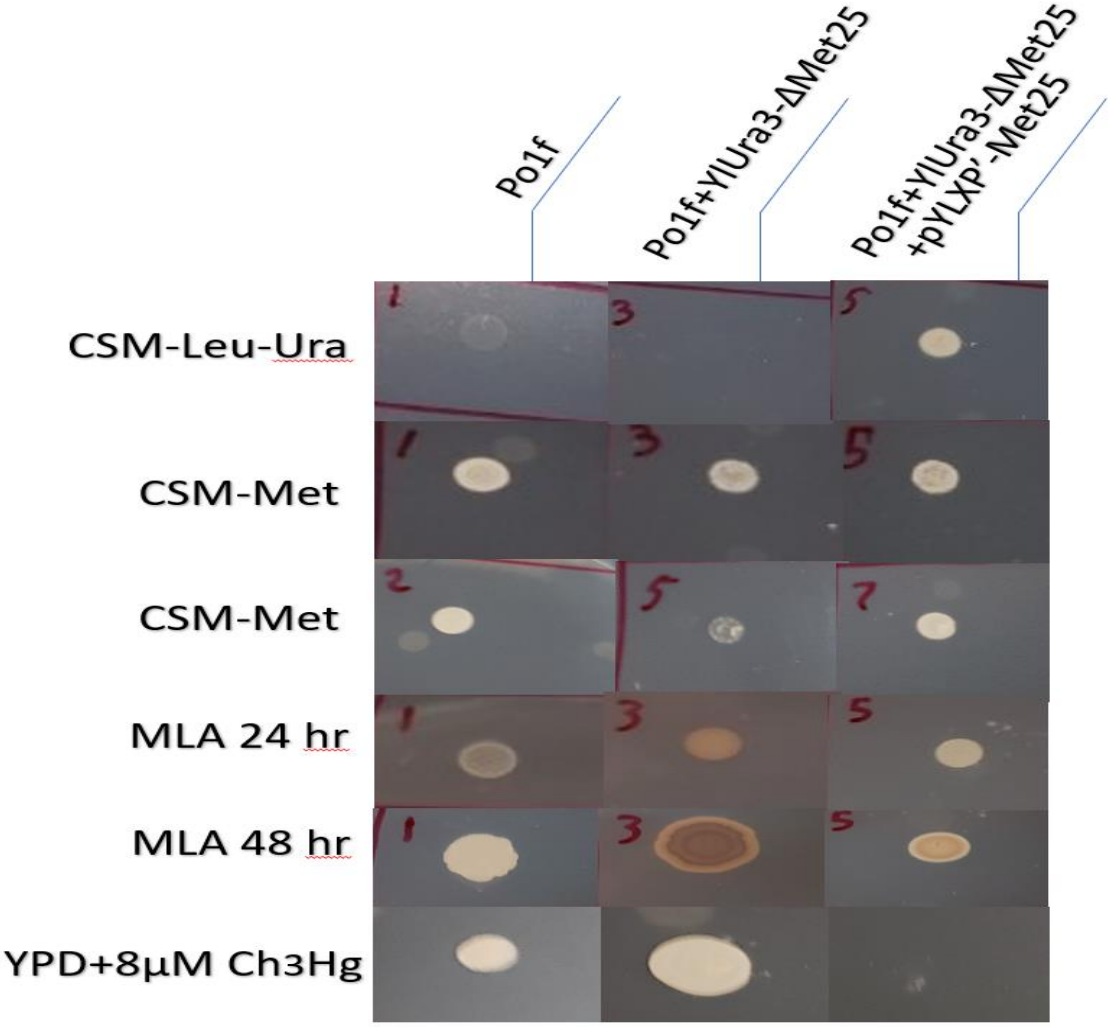

Fig. 2. This image contains the result various selective media assays. The columns left to right represent wildtype, mutant, and mutant containing restorative plasmid. The first row contains all strains growing on CSM-Leu-Ura. The second and third row contain all strains growing on CSM-Met, without and with cell washing, respectively. The fourth and fifth row demonstrates all strains growing on MLA, at 24 and 48 hours, respectively. The strains in the fifth row were grown on rich media containing 8mM methyl mercury.

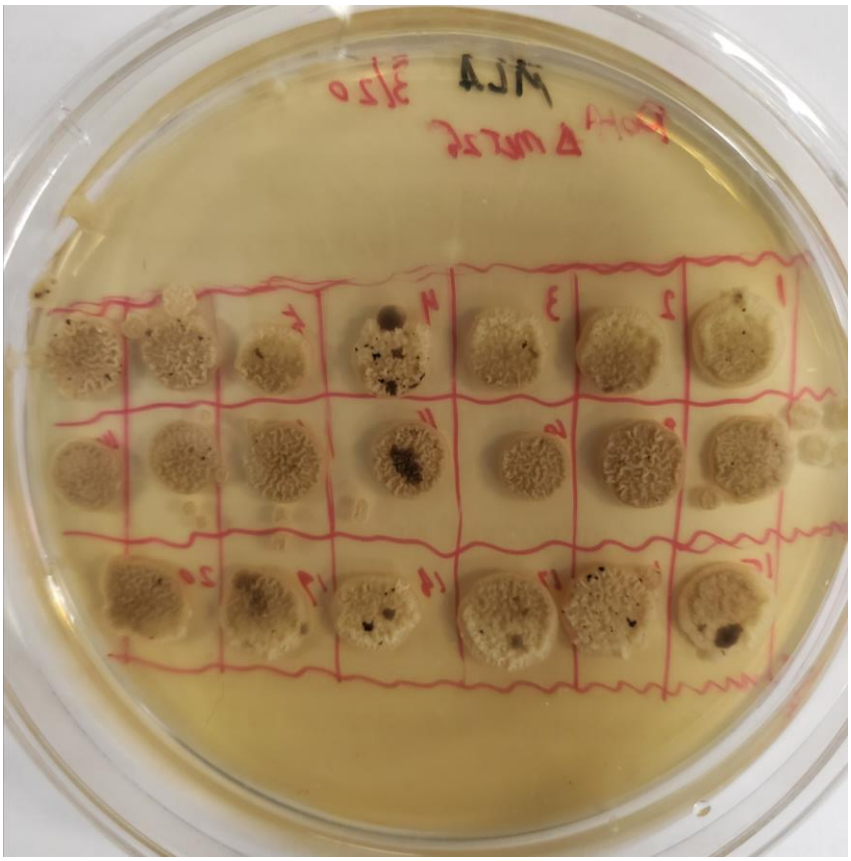

Fig. 3. Colonies from a successful transformation of Po1f with pYLXP'-AsCpf1-AsCrRNA-Met25 on CSM-Leu, subsequently spotted onto MLA.

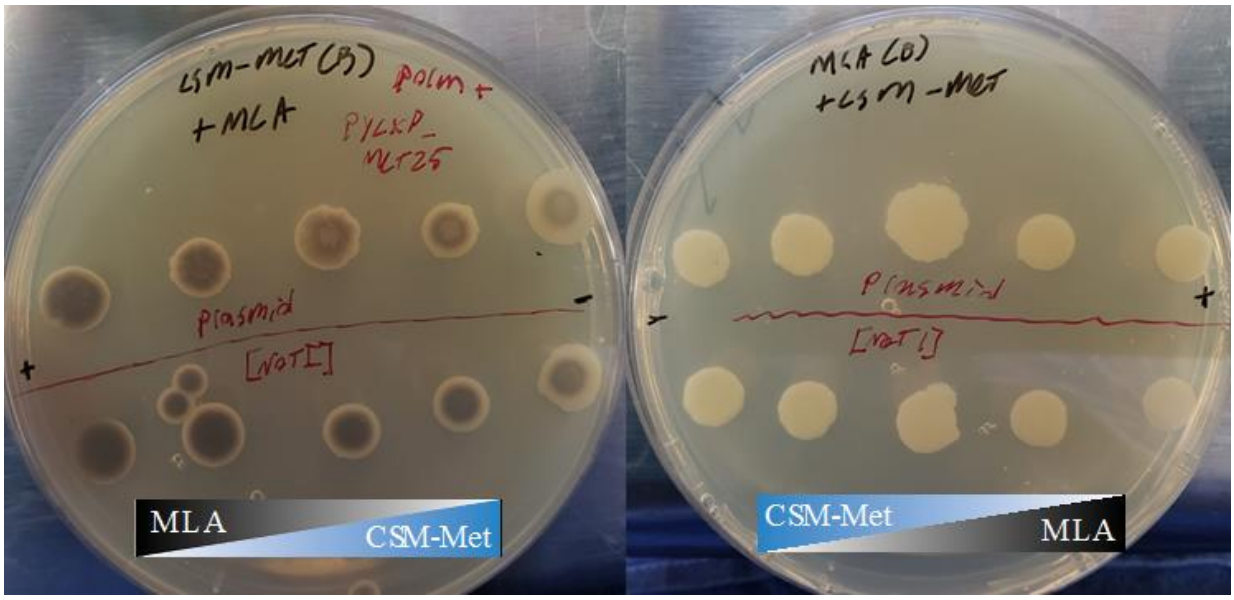

Fig. 4. On the left panel, CSM-Met on bottom, MLA on top, decreasing lead (Pb^{2+}) and methionine availability from left to right. On the right panel, MLA on bottom, CMS-Met on top, increasing lead (Pb^{2+}) and methionine availability from left to right. Colonies with plasmid-based MET25 complementation was above the red equator line, colonies with chromosomally integrated MET25 (*via Not*1 linearization) was placed below the red equator line.

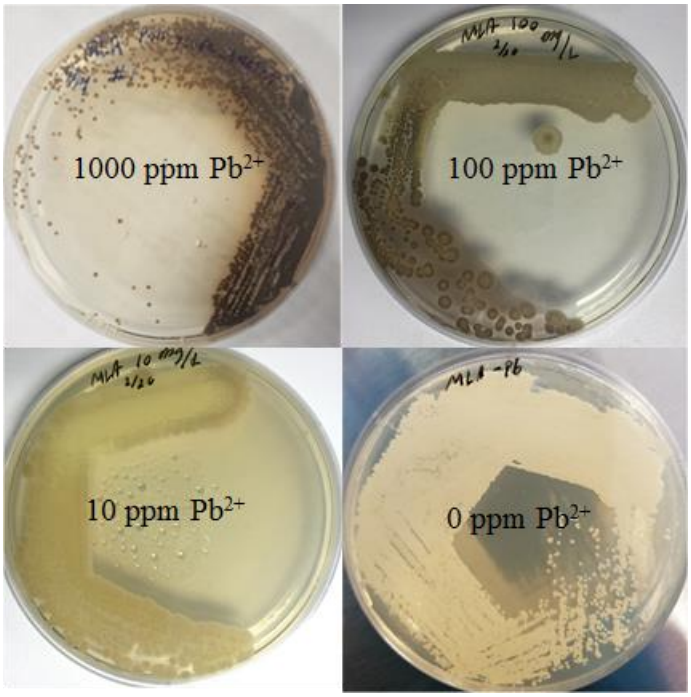

Fig. 5. MET25 deficient cells on MLA plates containing lead ranging from 0 ppm to 1000 ppm.

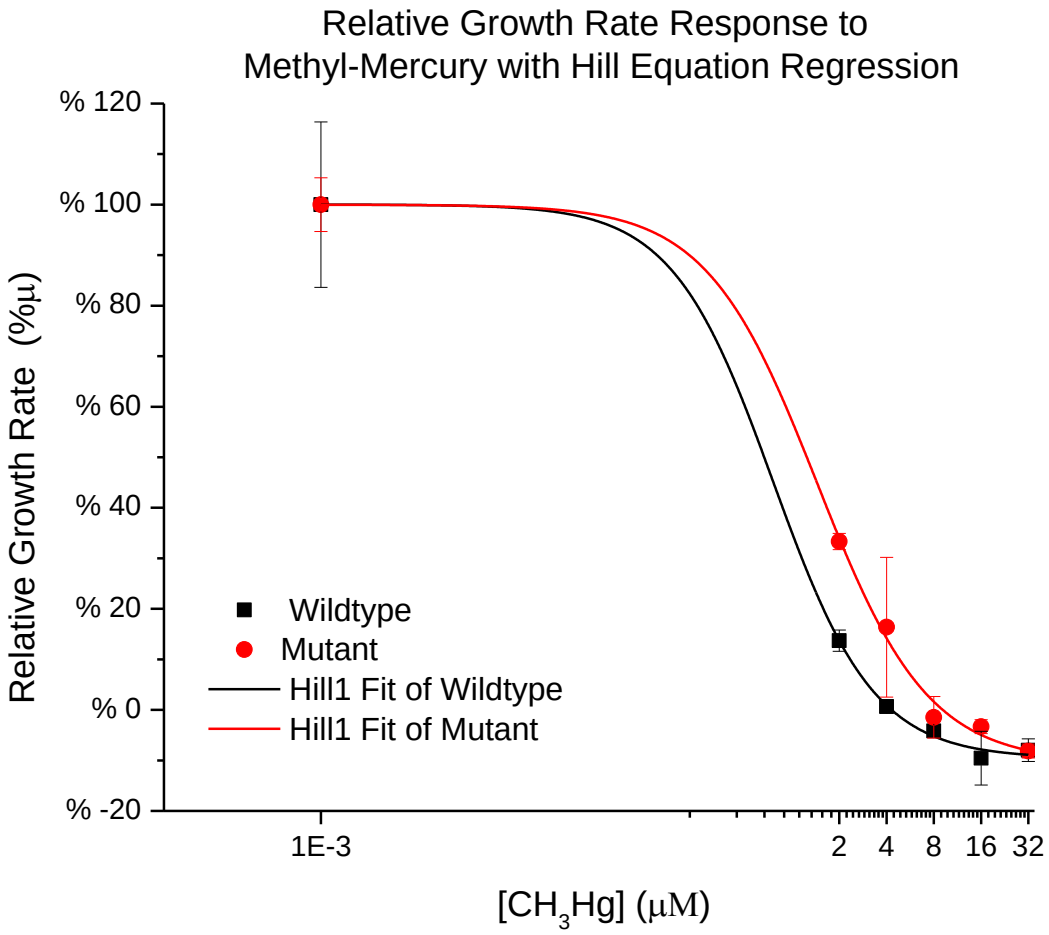

Fig. 6. Relative specific growth rate in the presence of methyl mercury is observed to correlate strongly with a Hill type equation. Points indicate biological triplicates where the lines represent regression curves. Black graphics indicate wildtype and red indicate mutants lacking MET25.