Comparative functional genomics identifies an iron-limited bottleneck in a *Saccharomyces cerevisiae* strain with a cytosolic-localized isobutanol pathway

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**ABSTRACT**

Metabolic engineering strategies have been successfully implemented to improve the production of isobutanol, a next-generation biofuel, *in Saccharomyces cerevisiae*. Here, we explore how two of these strategies, pathway relocalization and redox cofactor-balancing, affect the performance and physiology of isobutanol producing strains. We equipped yeast with isobutanol cassettes which had either a mitochondrial or cytosolic localized isobutanol pathway and used either a redox-imbalanced (NADPH-dependent) or redox-balanced (NADH-dependent) ketol-acid reductoisomerase enzyme. We then conducted transcriptomic, proteomic and metabolomic analyses to elucidate molecular differences between the engineered strains. Pathway localization had a large effect on isobutanol production with the strain expressing the mitochondrial-localized enzymes producing 3.8-fold more isobutanol than strains expressing the cytosolic enzymes. Cofactor-balancing did not improve isobutanol titers and instead the strain with the redox-imbalanced pathway produced 1.5-fold more isobutanol than the balanced version, albeit at low overall pathway flux. Functional genomic analyses suggested that the poor performances of the cytosolic pathway strains were in part due to a shortage in cytosolic Fe-S clusters, which are required cofactors for the dihydroxyacid dehydratase enzyme. We then demonstrated that this cofactor limitation may be partially recovered by disrupting iron homeostasis with a fra2 mutation, thereby increasing cellular iron levels. The resulting isobutanol titer of the fra2 null strain harboring a cytosolic-localized isobutanol pathway outperformed the strain with the mitochondrial-localized pathway by 1.3-fold, demonstrating that both localizations can support flux to isobutanol.

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1. Introduction

Isobutanol is a branched four-carbon alcohol targeted by many as a biofuel for light-duty engines and as a precursor to jet and diesel fuels via catalytic upgrading [1]. Relative to ethanol, isobutanol has a higher energy density, a lower vapor pressure, and a higher flash point, making it a better option for spark-ignition engines. Isobutanol is also less corrosive and less hygroscopic, making it a better biofuel for current piping infrastructure [2]. Isobutanol biosynthesis is redox balanced with glycolysis and can therefore be made via fermentation. Saccharomyces cerevisiae, the workhorse for industrial fermentations, is therefore a suitable host for its production. While yeast have a natural ability to ferment carbohydrates, the first half of the pathway converts pyruvate to α-ketoisovalerate (KIV) by mitochondria-localized ILV enzymes: acetoacetate synthase (ALS, encoded by ILV2), a NADPH-dependent ketol-acid reductoisomerase (KARI, encoded by ILV3), and a 2Fe–2S cluster-requiring dihydroxyacid dehydratase (DHAD, encoded by ILV3). The second part of the pathway, the Ehrlich pathway, converts KIV to isobutanol by promiscuous cytosolic enzymes: α-ketoacid decarboxylase (KDC, encoded by pyruvate decarboxylases, PDC1 PDC5 or PDC6, or phenylpyruvate decarboxylase, ARO10) and a NADH-dependent alcohol dehydrogenase (ADH, encoded by ADH1-5) (Fig. 1a). S. cerevisiae’s native isobutanol production is low (~1% of the theoretical maximum yield), so substantial work is needed to make isobutanol the primary fermentation product [3-6].

S. cerevisiae synthesizes isobutanol from pyruvate via enzymes involved in valine biosynthesis and the Ehrlich pathway. The first half of the pathway converts pyruvate to α-ketoisovalerate (KIV) by mitochondria-localized ILV enzymes: acetoacetate synthase (ALS, encoded by ILV2), a NADPH-dependent ketol-acid reductoisomerase (KARI, encoded by ILV3), and a 2Fe–2S cluster-requiring dihydroxyacid dehydratase (DHAD, encoded by ILV3). The second part of the pathway, the Ehrlich pathway, converts KIV to isobutanol by promiscuous cytosolic enzymes: α-ketoacid decarboxylase (KDC, encoded by pyruvate decarboxylases, PDC1 PDC5 or PDC6, or phenylpyruvate decarboxylase, ARO10) and a NADH-dependent alcohol dehydrogenase (ADH, encoded by ADH1-5) (Fig. 1a). S. cerevisiae’s native isobutanol production is low (~1% of the theoretical maximum yield), so substantial work is needed to make isobutanol the primary fermentation product [3-6].

The spatial segregation of the native pathway enzymes, split between the mitochondria and the cytosol, may be a limiting factor for synthesizing isobutanol at high levels. The segregation can be overcome by localizing the five-step pathway into a single compartment: mitochondrial localization is achieved by adding a mitochondrial localization sequence (MLS) to the genes in the Ehrlich pathway (PDC1/5/6 or ARO10 and ADH1-5), and cytosolic localization is achieved by removing the MLS sequence from the genes in the valine biosynthesis pathway (ILV2, ILV5, and ILV3) [5,6]. However, the role that localization plays in isobutanol production is obscured by conflicting reports in the literature as both pathway localizations have achieved similar isobutanol titers and yields (Table 1). It has been shown that a strain with mitochondrial-localization benefits from having a higher local concentration of enzyme and pathway intermediates [5]. On the other hand, it has been proposed that cytosolic localization would be preferable in the context of industrially relevant conditions such as high glucose concentrations or anaerobic growth. This is in part because the mitochondria are known to enter a minimal energy-requirement mode under those conditions [7,8].

A second limitation in the isobutanol pathway is the redox cofactor imbalance that exists between glycolysis and isobutanol fermentation; glycolysis produces two NADH per molecule of glucose, while isobutanol fermentation consumes one NADPH by ILV5 and one NADH by ADH. This results in an NADPH shortage and NADH excess. One strategy to resolve this imbalance is to use a heterologous NADH-dependent KARI, such as the E. coli KARI variant, ILVc605, that was previously engineered with improved specificity towards NADH [9,10]. Another strategy is to increase the supply of NADPH by introducing a transhydrogenase-like shunt (NADH + NADP+ → NAD+ + NADPH) into S. cerevisiae [11]. Both strategies have been successful in improving

![Fig. 1. Native and engineered S. cerevisiae isobutanol pathways. a) Shows the endogenous isobutanol synthesis pathway from pyruvate. The first half of the pathway converts pyruvate to α-ketoisovalerate (KIV) by the mitochondrial-localized valine biosynthesis enzymes Ilv2p, Ilv5p, and Ilv3p. The second part of the pathway, the Ehrlich pathway, converts KIV to isobutanol by promiscuous cytosolic-localized decarboxylase enzymes Pdc1/5/6 or Aro10p and Adh1-5p. b) Mitochondrial-localized isobutanol pathway (mIBA) consists of codon-optimized variants of S. cerevisiae Ilv2p, S. cerevisiae Ilv3p, mitochondrial targeted S. cerevisiae Aro10p, mitochondrial targeted L. lactis AdhA29C8p, and either S. cerevisiae Ilv5p or mitochondrial targeted E. coli IlvC605p for the redox cofactor imbalanced and balanced pathways respectively (red enzymes). c) Cytosolic-localized isobutanol pathway (cIBA) consists of codon-optimized variants of S. cerevisiae Ilv22-54p, S. cerevisiae Ilv52-54p, S. cerevisiae Aro10p, L. lactis AdhA29C8p, and either S. cerevisiae Ilv52-54p or E. coli IlvC605p for the redox cofactor imbalanced and balanced pathways respectively (red enzymes). For both the mIBA and cIBA pathways, the endogenous/native enzymes (Ilv2p, Ilv5p, Ilv3p, Aro10p, Pdc1/5/6p, Adh1-5p, and Bat1p) in black remain in each strain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)](null)
isobutanol titers, demonstrating the importance of having a redox cofactor-balanced pathway (Table 1).

In this work, we aimed to provide insight into how pathway localization and redox cofactor-balancing affect the performance and physiology of isobutanol producing strains. We conducted a functional genomics analysis to elucidate differences between \textit{S. cerevisiae} strains equipped with DNA cassettes that localized isobutanol biosynthesis to specific compartments, i.e. mitochondria vs. cytosol, and included either a NADPH-dependent (cofactor-imbalanced) or NADH-dependent (cofactor-balanced) KARI enzyme. Here, we report that pathway localization had a greater effect on isobutanol production than redox-balancing; the strain harboring the mitochondrial-localized isobutanol pathway outperformed the cytosolic version by 3.8-fold. The main limitation in the cytosolic-localization is the supply of the 2Fe–2S cluster cofactor for the DHAD enzyme; however, we found that this limitation may be partially overcome by increasing the availability of iron in the cell by perturbing iron homeostasis. Our findings contribute towards improving the metabolic engineering designs for building further improved isobutanol producing strains.

2. Materials and methods

2.1. Yeast media

Complex lab media for culturing, YP, consisted of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose (YPD). 200 g/L MES, 100 g/L dextrose, 4 mL/L 250X tween/ergosterol stock (62.5 mL Tween80 + 625 mg Ergosterol in 187 mL 95% EtOH) and pH adjusted to 5.5. Defined synthetic complete media contained 6.7 g/L yeast nitrogen base (YNB) without amino acids with ammonium sulfate, 1 g/L drop-out mix without yeast nitrogen base, and 20 g/L dextrose.
of 672 bp upstream of the ScTDH3 protein-coding sequence, \( T_{PEF} \) consisted of 350 bp downstream of the ScTEF1 protein-coding sequence, \( T_{PEB} \) consisted of 577 bp upstream of the ScTEF1 protein-coding sequence, \( T_{PTEB} \) consisted of 380 bp downstream of the ScTUB1 protein-coding sequence. The transcriptional units (promoter–gene–terminator pairs) for the five genes were then cloned in the above order into the SacI-KpnI polynucleotides of a modified pRS426 plasmid containing PygMX4 as the selection marker in place of URA3 [26]. Homology arms to the HO locus (HO-L and HO-R) were inserted 5′ and 3′ of the isobutanol pathway cassette to facilitate genome insertion by homologous recombination [27]. The HO-R homology arm also included the LoxP-KanMX-LoxP selection marker, which was inserted 3′ of ScTUB1 protein-coding sequence [28]. For expressing ARO10 and adhA29c8s, the mitochondrial coding sequence, the COX4-1 MLS was synthesized in-frame at the 5′ ends of codon-optimized ARO10 and adhA29c8s. The full length isobutanol pathway cassette containing mitochondrial-localized enzymes and flanking HO arms (GenBank: MZ541859) was excised from the plasmid by digestion with SacI-HF and KpnI-HF, purified and transformed into the WT strain. After selection on YPD + GenBank, PCR and Sanger sequencing were used to confirm the insertion of the isobutanol pathway cassette into the HO locus. The LoxP-KanMX-LoxP selection marker was then excised by Cre recombinase to generate the final mIBA strain [28]. The inserted isobutanol pathway cassette was amplified by PCR with multiple primer sets from mIBAILV genomic DNA (gDNA) and confirmed for complete insertion by Sanger-sequencing (Fig. S1a). To generate the cIBA strain, CRISPR/Cas9 was used to delete the MLS at the N-terminus of the engineered isobutanol pathway enzymes in mIBAILV (Fig. S1b). In brief, sgRNA sequence targeting the mLS of each synthetic isobutanol pathway enzyme were identified by CRISpy-pop [29] and cloned into the pXPHOS plasmid as described previously [30]. sgRNA targeting sequences are as follows: ILV2, CCTAATAGCGAAGTTCTCA; ILV3, AGTAGTAGAATTGCTAG; ILV5, GCAGATGACTTGGCTGTT; MLS-adjA29c8s and MLS-aro10A, AAATCCTGTGACGTCTCA. Each mitochondrial-localized gene in the synthetic isobutanol pathway was sequentially replaced by transforming the pXPHOS plasmid containing a single sgRNA to one gene along with the appropriate PCR repair template of the gene lacking the MLS in the following order: ILV5 \( - \) ilvC29c8s, MLS-aro10A \( - \) MLS-adjA29c8s simultaneously \( - \) ARO10 and adhA29c8s, ILV3 \( - \) ILV32-19, ILV2 \( - \) ILV22-34. To generate repair templates with the cytosol-localized isobutanol pathway enzymes, individual genes were first cloned into the TOPO vector and then the appropriate MLS was removed (ILV22-34, ILV32-19 and ILV52-48, ARO10MLS, and adhA29c8s, adjA29c8s, MLS [6]) by restriction digestion/PCR-aided Gibson assembly and confirmed with Sanger-sequencing. These vectors were then used as the PCR repair template which contained part of the promoter and part of the gene lacking the MLS. The repair template for replacing ILV5 with ilvC29c8s was generated by amplifying the E. coli ilvC29c8s CDS from pTB1A [31] and cloning it in place of the ILV52-48 CDS in the TOPO vector; this vector was Sanger-sequenced and used for the PCR repair template in the same manner as described earlier. To generate the cIBA0ILV5 strain, the cIBA0ilvC8s strain was transformed with the pXPHOS plasmid containing an sgRNA to ilvC8s (AGTTTATAGCGAAGCGTGA) and a PCR repair template of the ILV52-48 (Fig. S1c.). To generate the mIBA0ilvC8s strain, the mIBA0ILV5 strain was transformed with the pXPHOS plasmid containing an sgRNA to codon-optimized ILV5 (GCAGAATCTGAGCGTGA) and a PCR repair template containing MLS-ilvC8s (Fig. S1d). For the cIBA0ILV5, cIBA0ilvC8s and mIBA0ilvC8s strains, DNA sequence changes where CRISPR/Cas9 editing occurred were confirmed by Sanger-sequencing. Deletions of codon-optimized ILV3, native ILV3 and/or native FRA2 from the cIBA0ILV5, mIBA0ILV5, and cIBA0ilvC8s strains were performed by integration of PCR products generated from LoxP-KanMX-LoxP (pUG6) or LoxP-HphMX-LoxP (pUG7) plasmid templates and primers containing 40–60 bp of homology flanking the targeted genes [32]. PCR products were purified and transformed into the appropriate strains and selected for growth on the appropriate antibiotic. Gene deletions were confirmed by PCR of gDNA and Sanger-sequencing.

### 2.3. Fermentation growth conditions and sample collection

For aerobic growth, starter cultures were grown in a shaking incubator at 30 °C. For anaerobic growth, media was allowed to degas in a 30 °C Coy anaerobic chamber (10% H2, 10% CO2, and 80% N2) for 12 h prior to use and cultures were grown with stir bars on a magnetic stir plate to prevent flocculation. Fermentations for multi-omic sampling were performed in biological triplicate. Yeast cells were grown aerobically in YPD until stationary phase (OD600 ~12 h). The cultures were then shifted to minimal medium and anaerobic conditions by diluting the culture to OD600 0.3 in minimal medium and allowed to reach exponential phase ~8.5 h. Working cultures, which were used for sampling, were then inoculated from the shifted culture to OD600 0.2 in minimal medium and allowed to grow anaerobically for 48 h. Cell growth was monitored by taking OD600 measurements with the Beckman DU720 spectrophotometer. Samples for end-product analysis were taken by collecting 2 mL of culture supernatant. Samples for protein isolation were obtained by collecting and flash freezing ~1 × 1012 cells. Similarly, samples for RNA isolation were obtained by collecting and flash freezing the equivalent of 25 mL of cells at an OD600 of 1. Samples for intracellular metabolite quantification were collected inside the anaerobic chamber by vacuum filtrations of culture through 0.45 μm hydrophilic nylon filters. Filters with the retained cells were immediately placed cell-side down in 1.5 mL of extraction solvent (40 vol% acetonitrile, 40 vol% methanol, 20 vol% water) kept on dry ice to quench metabolism and extract metabolites.

### 2.4. End-product analysis

#### 2.4.1. Determination of isobutanol concentration by automated headspace GC/MS

The equipment used included the following: an Agilent 7890A GC (Agilent Technologies, Inc. Palo Alto, CA); a LPA3 autosampler and sample preparation system equipped with a heated agitator/stirrer and heated headspace sampling syringe (Agilent Technologies, Inc. Palo Alto, CA); and a Pegasus 4D ToF-MS (Leco Corp., Saint Joseph, Michigan). Typical analysis range is 0.065 mM–8.4 mM isobutanol and uses an aliquot volume of 500 mL and 2-Methylpropyl-d9 alcohol (as internal standard). Instrument run control and conditions are set by the Chromatof® software (version 4.72.0.0) provided with the Pegasus 4D GCxGC ToF MS system. Samples were incubated at 70 °C for 5 min in the heated agitator set to 350 rpm. 0.5 mL of the headspace is then sampled by the autosampler with a 2.5 mL gastight syringe heated to 75 °C and injected into the GC system. The sample was withdrawn at 100 μL/s and injected into the GC at 1000 μL/s. The syringe was purged with nitrogen gas for 0.5 min prior to the next injection. The analytical capillary GC column was a Stabilwax-DA® (Restek, Inc. Bellefonte, PA) length 30 m, 0.25 mm ID, 0.25 μm film thickness. Helium was used as a carrier gas with a pressure corrected constant flow rate of 1 mL/min. The GC inlet was fitted with a 4 mm deactivated glass liner and held at 250 °C throughout the run. The inlet split ratio was set to 50:1. The GC oven was initially set to 50 °C and held for 1 min then increased to 200 °C at 40 °C/min and held at 200 °C for 5 min. The filaments of the mass spectrometer were turned on 42.5 s after injection and 10 spectra/sec were recorded from m/z 10 to m/z 250. The MS source temp was 200 °C, electron energy set to 70 eV, and the detector voltage was adjusted to approximately 50 V above the minimum voltage determined by the instrument tune check procedure. The peak area of isobutanol was measured using the extracted ion chromatogram of m/z 43 and the peak area of 2-Methylpropyl-d9 alcohol was measured from the extracted ion chromatogram of m/z 46. An unplugged t-test was performed to determine statistical significance.
2.4.2. Analysis of fermentation supernatants by HPLC-RID

End-product analytes (glucose, ethanol, glycerol, lactate, and acetate) were measured with an analytical system consisting of an Agilent 1260 Infinity HPLC system (Agilent Technologies, Inc., Palo Alto, CA) with a quaternary pump, chilled (4°C) autosampler, vacuum degasser, refractive index detector, and a Aminex HPX-87H column with a Cation-H guard column (BioRad, Inc. Hercules, CA; 300 × 7.8 mm, cat# 125–0140). Operating parameters were as follows: 0.02 N H2SO4 mobile phase, 0.500 mL/min flow rate, 50 °C column temperature, 50 °C detector temperature, 28 min run time, and a 50 μL injection volume. Instrument control, data collection and analysis/calculation are done using Chem Station V. B04.03 software (Agilent Technologies, Inc., Palo Alto, CA). An unpaired t-test was performed to determine statistical significance.

2.5. Intra- and extra-cellular metabolomics preparation, quantification, and analysis

After collecting all samples at a given time point, the filter disks in extraction solvent were removed from the anaerobic chamber and the cells were washed off the filter into the solvent. The entire suspension was transferred into a centrifuge tube and centrifuged for 5 min at 16,000×g at 4 °C to remove cellular debris. The supernatant was collected in another centrifuge tube and immediately prepped for quantification.

The extraction solvent containing intracellular metabolites was dried under N2 and the metabolites were resuspended in Solvent A (97:3 H2O: methanol with 10 mM tributylamine adjusted to pH 8.2 by addition of 10 mM acetic acid). Samples were analyzed by LC-MS as described previously [33,34]. Data analysis was performed using MAVEN software [35,36] and compounds were identified by retention time (matched to pure standards). "Peak Area Top" values were extracted and normalized by OD600 equivalent of sample injected into the LC-MS. Extracellular metabolite samples for LC-MS analysis were diluted to appropriate concentration, then analyzed using the above method. An unpaired t-test was performed to determine statistical significance.

2.6. Transcriptomic sample preparation, library construction, sequencing, and analysis

Total RNA was extracted with hot phenol lysis [37], DNA was digested with Turbo-DNase (Life Technologies, Carlsbad, CA) for 30 min at 37 °C, and RNA was precipitated with 2.5 M LiCl for 30 min at 20 °C. In brief, RNA-Seq libraries were generated using the Illumina TruSeq® Stranded mRNA HT kit, AMPure XP bead for PCR purification (Beckman Coulter, Indianapolis, IN), and SuperScriptI reverse transcriptase (Invitrogen, Carlsbad, CA) as described in the Illumina kit. Single-end 50-bp reads were generated using an Illumina NovaSeq 6000 at the University of Wisconsin-Madison Biotechnology Center. Reads were processed with Trimmomatic version 0.3 [38] and mapped to the S288C genome (with foreign mutant sequences added) using Bowtie 2 version 2.2.2 with default settings [39]. Read counts were calculated using HTseq version 0.6.0 [40]. edgeR version 3.6.8 [41] was used to perform differential gene analysis, taking a Benjamini and Hochberg false discovery rate (FDR) of <0.05 as significant [42]. Raw sequencing counts were normalized using Trimmed Means of M-values (TMM). Samples were clustered using Cluster 3.0 [43] and visualized in Java TreeView [44], and functional GO term enrichment was performed using SetRank [45] with an FDR set to <0.05 for significance.

2.7. Label-free quantitative proteomics preparation, quantification, and analysis

Yeast pellets were resuspended in 150 μL of 6 M guanidine, 100 mM Tris (pH 8) and boiled for 5 min at 100 °C. Methanol was then added to 90% to precipitate proteins, and the samples were centrifuged for 5 min at 9,000 G. The supernatant was discarded, and the protein pellet was resuspended in lysis buffer (8 M urea, 100 mM Tris pH 8, 20 mM TCEP, 80 mM Chloroacetamide). LysC was added to an estimated 50:1 protein to enzyme ratio and incubated for 4 h at room temperature. The samples were diluted with 100 mM Tris to a urea concentration of 1.5 M. Trypsin was added to an estimated 50:1 protein to enzyme ratio and incubated overnight at room temperature. Samples were desalted with Strata C18 solid phase extraction cartridges and dried in a vacuum centrifuge. Peptide concentration was then determined from a NanoDrop One spectrophotometer before re-suspending in 0.2% formic acid and injecting onto the mass spectrometer.

Samples were analyzed using an LC-MS/MS instrument comprising an Orbitrap Eclipse Tribrid mass spectrometer and UltiMate 3000 RSLCxano liquid chromatography system (Thermo Fisher Scientific). Mobile phase A consisted of 0.2% formic acid in water and mobile phase B consisted of 0.2% formic acid in 80% acetonitrile. Peptides were loaded in 0% B and separated at a flow rate of 310 nL/min over a 120 min gradient of increasing % B. Peptides were injected onto a 1.7 μm C18 column (75 μm i.d.) packed in-house to a length of 30 cm [46] and heated to 50 °C. Survey scans of peptide precursors were collected every second from 300 to 1350 Th with an AGC target of 1,000,000 and a resolution of 240,000 in the orbitrap. Precursors were isolated from a 0.5 Th window in the quadrupole and HCD MS/MS scans at 35% collision energy were collected in the ion trap with an AGC target of 35,000 from 150 to 1350 Th.

The resulting LC-MS/MS proteomics data were processed using MaxQuant [47] software version 1.5.2.8 and searched against a Saccharomyces cerevisiae database (with foreign mutant proteins added) downloaded from Uniprot. The digestion enzyme was set to Trypsin/P with up to two missed cleavages, and oxidation of methionine and protein N-terminal acetylation were set as variable modifications. Cysteine carbamidomethylation was set as a fixed modification. Label-free quantification was enabled with a minimum ratio count of 1, and the match between runs feature was utilized to decrease missing data values within the dataset. Peptides were filtered to a 1% FDR and combined to protein groups based on the rules of parsimony. Differential protein analysis was done taking a Benjamini and Hochberg false discovery rate (FDR) of <0.05 as significant [42]. Functional enrichment was assessed using the FunSpec database [48].

2.8. Petite frequency

To assess the propensity of different strains to form petite colonies due to loss of respiratory competence each strain was revived on YPD media and streaked for single colonies on YPG (2% glycerol) media to ensure all starting cells could respirate. Single colonies were used to inoculate 3 independent 5 mL YPD cultures, which were grown rolling overnight at 30 °C. Each culture was plated for single colonies on YPDG (0.1% dextrose, 3% glycerol) media to differentiate petite and respiring colonies and colonies were counted after 3 days of growth at 30 °C.

3. Results and discussion

3.1. Engineered isobutanol producing strains

In order to evaluate the effects of pathway localization and redox cofactor-balancing on isobutanol production, we constructed four engineered strains: a mitochondrial-localized isobutanol pathway with a NADPH-dependent Ilv5p KARI, mIBA6E6; a mitochondrial-localized isobutanol pathway with a NADH-dependent IlvC6E6p KARI, mIBA6E6; a cytosolic-localized isobutanol pathway with a NADPH-dependent Ilv5p KARI, cIBA6E6; and a cytosolic-localized isobutanol pathway with a NADH-dependent IlvC6E6p KARI, cIBA6E6 (Fig. 1b and c, Table S1 / Fig. S1, Materials and methods 2.2). DNA cassettes for expressing each pathway were assembled from genes previously tested [5], including those encoding an alcohol dehydrogenase (adhA29C8), from
Lactococcus lactis (25)), an acetolactate synthase (ILV2, from S. cerevisiae), a dihydropyrimidin dehydratase (ILV3, from S. cerevisiae), a α-ketoacid decarboxylase (ARO10, from S. cerevisiae), and a ketol-acid reductoisomerase (ILV5, from S. cerevisiae or iblC<sup>66</sup>, from E. coli (31)). Each open reading frame was codon-optimized, synthesized commercially, and cloned in frame with promoters (P) and terminators (T) resulting in the following pairs: P<sub>ADH1</sub>-ILV2-T<sub>CYC1</sub>; P<sub>PGK1</sub>-ILV3-T<sub>TEF1</sub>; P<sub>TEF1-adrB<sup>29C6</sup>-T<sub>TUB1</sub>; P<sub>TUB1</sub>-ARO10-T<sub>TEF1</sub>; P<sub>TEF1</sub>-ilv<sup>C66</sup>-T<sub>TUB1</sub> (Materials and methods 2.2). Mitochondrial localization was achieved by adding DNA encoding the previously characterized Cox4p N-terminal MLS [5] to the N-terminus of genes which natively localized to the cytosol (MLS-ARO10, MLS-adrB<sup>29C6</sup>, and MLS-ilv<sup>C66</sup>). Conversely, cytosol targeting was achieved by removing sequences encoding previously identified MLS from genes which natively localized to the mitochondria (ILV2<sup>2-54</sup>, ILV3<sup>2-19</sup>, and ILV5<sup>2-48</sup>) (6). DNA cassettes containing each of the five genes were assembled and integrated into the S. cerevisiae genome at the HO locus via homologous recombination. When constructing the mIBA<sup>hC66</sup> strain we uncovered an unexpected petite phenotype. The frequency at which the petite colonies arose in the mIBA<sup>hC66</sup> strain was 166-fold that of the wild-type (WT) strain (Table S2). All other engineered strains had a low petite frequency consistent with natural S. cerevisiae isolates (49,50). The mIBA<sup>hC66</sup> strain was not included in further studies due to this irreversible fitness defect.

3.2. Engineered strains produce varied amounts of isobutanol

A fermentation experiment was performed to evaluate and compare the physiological and molecular phenotypes of the engineered strains and WT. Each strain was cultivated anaerobically at 30 °C in minimal medium containing 100 g/L glucose. We collected samples every 2 h to monitor growth, glucose consumption, isobutanol production, and ethanol production. Samples for functional genomics analyses (transcriptomic, proteomic and metabolomic) were collected at 4, 10, and 26 h post inoculation to represent the different phases of cell growth: early-exponential phase, mid-exponential phase, and early-stationary phase (Fig. 2). The three engineered strains grew slower than the WT strain (Fig. 2); doubling times during the exponential phase of the mIBA<sup>LVS</sup>, cIBA<sup>LVS</sup> and cIBA<sup>hC66</sup> strains were 1.1, 1.3, and 1.3-fold larger than the WT strain, respectively. All strains completely consumed the glucose provided and reached similar final OD<sub>600</sub> (5.3–5.6) values by the end of the 48 h fermentation. All strains produced ethanol titers of 45–47 g/L by 44 h of the fermentation corresponding to yields over 90% of the theoretical maximum (Fig. 2). These metrics indicate that ethanol remains the dominant fermentation product and that pathway localization and cofactor-balance alone were not the limiting factor in isobutanol fermentation.

Isobutanol production varied significantly among the engineered strains. The cIBA<sup>LVS</sup> and cIBA<sup>hC66</sup> strains produced isobutanol at levels less than or equal to WT; at 38 h, the isobutanol titers were 37, 46, and 31 mg/L in the WT, cIBA<sup>LVS</sup>, and cIBA<sup>hC66</sup> strains, respectively. The cIBA<sup>LVS</sup> strain produced 1.5-fold (P < 5E-5) more isobutanol than the cIBA<sup>hC66</sup> strain indicating that redox cofactor-balancing is not a limiting factor for driving flux to isobutanol synthesis in these strains and NAD⁺ is preferentially regenerated through ethanol fermentation. The highest isobutanol titer was achieved by the mIBA<sup>LVS</sup> strain, which produced 170 mg/L at 38 h, corresponding to a yield of 1.8 mg isobutanol/g glucose. This metric is similar to other isobutanol overproducing strains grown under anaerobic conditions [21]. In summary, the mIBA<sup>LVS</sup> strain outperforms the other engineered strains and had a 3.8-fold (P < 5E-6) higher isobutanol titers than the cIBA<sup>LVS</sup> strain.

3.3. Strains with cytosolic-localized isobutanol pathway have an altered transcriptome and proteome

We next assessed the molecular phenotypes of the engineered strains and WT using the functional genomics samples collected during the above-mentioned fermentation experiment. We identified and collected data for 59 intracellular metabolites using LC-MS, 34 extracellular metabolites (28 LC-MS and 6 HPLC/GC), 4339 proteins by LC-MS/MS proteomics, and 6434 genes by RNA-seq (File S1–S4, Data availability).

The transcriptome and proteome data collected from our top isobutanol producer, mIBA<sup>LVS</sup>, was more similar to WT than the cIBA<sup>hC66</sup> and cIBA<sup>LVS</sup> strains which both displayed a larger number of differentially expressed RNA and proteins (Fig. 3a, Table S3). The cIBA<sup>hC66</sup> and cIBA<sup>LVS</sup> strains shared 175 of the same differentially expressed genes, which were enriched for oxidation-reduction processes, mitochondrial respiration, and TATA-containing genes (Fig. 3b). Hierarchical clustering of the data was also performed, and the strains clustered together in a distinct clade within each timepoint (Fig. 3c). Taken together, we concluded that the cIBA<sup>hC66</sup> and cIBA<sup>LVS</sup> strains have functionally similar transcriptomes and that a cytosolic-localized isobutanol pathway induces gene expression changes in specific biological processes.

The metabolite dataset showed that all engineered strains had significantly altered levels of aromatic amino acid biogenesis intermediates (Fig. S2 and Fig. S3). We suspect this was due to over-expression of the synthetic KDC enzyme, Aro10p, in our engineered strains enhancing aromatic amino acid degradation flux (Fig. S3). The depletion of phenylalanine (Phe) and tyrosine (Tyr) did not cause any unexpected changes in cell growth as supplementation with 0.1% (w/v)
Phe and Tyr yielded no improvement in growth rate (Fig. S4). Metabolites in the IBA pathway were also dramatically altered but more so in the cIBA<sub>BC66</sub> and cIBA<sub>LVS</sub> strains when compared to WT (Fig. S2). The remainder of the manuscript will discuss conclusions we drew after integrating specific aspects of the functional genomics data.

### 3.4. Cytosolic-localized isobutanol pathway has a bottleneck at Ilv3p

We first examined data related to enzymes in the isobutanol pathway. To judge if each of our synthetic enzyme variants was expressed, the mRNA counts and protein abundance data were investigated. Normalized counts (RPKM) for all synthetic codon-optimized isobutanol genes (Ilv2, Ilv5, ILV5<sup>Δ2-19</sup>, IlvC<sup>6E6</sup>, ILV3, ILV3<sup>Δ2-19</sup>, MLS-<i>adhA</i><sup>29C8</sup>, <i>adhA</i><sup>29C8C</sup>, MLS-<i>aro10</i>, and <i>aro10</i>) were successfully detected in the transcriptomes of the engineered strains and the differences in isobutanol production we observed between the engineered strains can be a result of our synthetic variants being successfully translated and contributing to the protein pool. Overall, neither the mRNA counts nor the protein abundance data yielded an explanation for the differences in isobutanol production we observed between the engineered strains. We contribute the higher isobutanol production in the mIBA<sub>LVS</sub> strain to be the result of added activity from the mitochondrial pathway localization.

Next, we looked at the intracellular levels of the isobutanol pathway intermediates in our engineered strains and saw that 2-acetolactate (AL), 2,3-dihydroxyisovalerate (DHIV), and α-ketoisovalerate (KIV) were significantly altered compared to the WT strain. At 4 h, the cIBA<sub>LVS</sub> strain had a 23-fold (<i>P</i> < 5.0E-4), 16-fold (<i>P</i> < 5.0E-5), and 4.1-fold (<i>P</i> < 5.0E-5) increase in intracellular AL, DHIV, and KIV, respectively when compared to WT. Similarly, at 4 h, the cIBA<sub>BC66</sub> strain had a 22-fold (<i>P</i> < 5.0E-4), 8.6-fold (<i>P</i> < 0.0005), and 2.5-fold (<i>P</i> < 5E-4) increase in intracellular AL, DHIV, and KIV, respectively when compared to WT. This trend of elevated intracellular AL, DHIV, and KIV in the engineered strains was also seen at the other timepoints, 10 and 26 h, but to a lesser extent. The significant accumulation of AL and DHIV in these strains indicates that ILV3 is a rate-limiting step. This finding was also in agreement with a previous study where additional
copies of ILV3 were added to boost production of KIV from DHIV [15]. In contrast to the cIBAIlvC6E6 and cIBAILV5 strains, intracellular levels of DHIV in the mIBAILV5 strain were at lower levels compared to the WT strain; specifically, the level of DHIV was 2.8-fold ($P < 5E-4$), 4.5-fold ($P < 0.005$), and 3.2-fold ($P < 0.005$) lower when compared to WT at 4, 10, and 26 h respectively, which indicates mitochondrial-localized Ilv3p is not a bottleneck.

We hypothesized that the mitochondrial-localized Ilv3p is not a rate-limiting step in the mIBAILV5 strain because the required cofactor for the enzyme, a 2Fe–2S cluster, is more accessible in the mitochondria where its biogenesis begins. This is not the case for the cIBAIlvC6E6 and cIBAILV5 strains since the synthesis and delivery of the required 2Fe–2S cluster into the cytosolic-localized Ilv3p requires Fe–S cluster biogenesis machinery that spans multiple compartments (both the mitochondria and cytosol). Specifically, the mitochondrial iron sulfur cluster (ISC) machinery is responsible for generating the sulfur-containing intermediate (X–S), which is then exported to the cytosol via the ABC transporter Atm1p [53]. Atm1p was upregulated approximately 2.0-fold in the cIBAIlvC6E6 and cIBAILV5 strains at all time points when compared to WT (Fig. 5b). Atm1p overexpression is predicted to increase the number of available clusters in the cytosol by increasing the abundance of the X–S intermediate used by the CIA machinery for generating cytosolic Fe–S clusters [53]. We deleted the endogenous mitochondrial-localized ILV3 in the cIBAIlvC6E6 strain and performed a growth complementation assay to see if our synthetic cytosolic-localized Ilv3p could restore growth on synthetic complete medium lacking valine; indeed, the strain harboring only the cytosolic-localized Ilv3p grew on synthetic complete medium minus valine plates indicating it was functional in its non-native subcellular compartment (Fig. S5). In summary, the lower performance of the strain with the cytosolic-localized pathway was due to a rate-limiting step in the pathway at Ilv3p.

### 3.5. Proteomics analysis revealed a cytosolic-localized isobutanol pathway results in altered sulfur metabolism

To further explore our hypothesis that the Fe–S cluster requiring enzyme was the rate-limiting step in the cIBAIlvC6E6 and cIBAILV5 strains, we took a closer look at genes involved in Fe–S cluster synthesis. The protein levels of enzymes in the ISC and CIA machinery between the engineered strains and WT were relatively unchanged, with the exception of Atm1p, the mitochondrial X–S intermediate transporter (Table S5). Atm1p was upregulated approximately 2.0-fold in the cIBAIlvC6E6 and cIBAILV5 strains at all time points when compared to WT (Fig. 5b). Atm1p overexpression is predicted to increase the number of available clusters in the cytosol by increasing the abundance of the X–S intermediate used by the CIA machinery for generating cytosolic Fe–S clusters [53]. In the cytosol, excess Fe–S clusters are sensed by the iron responsive transcription factor, Yap5p. Yap5p can stably bind to 2Fe–2S
Grx4p were elevated in the cIBA GRX4 transcription of genes to help regulate iron storage including -clusters, inducing a conformational change that activates the transcription, cysteine biosynthesis, and siroheme biosynthesis pathways were assimilation [GO:0000103] (File S5). The protein abundance of enzymes, Ilv3p. The elevated levels of Atm1p in conjunction with the one of the time points with Tyw1p having the highest fold change of 1.7 at10 h when compared to WT (Fig. 5 b). This increase in Yap5p responsive genes indicated there was an increased number of 2Fe clusters in the cytosol of the cIBA strain. We next asked if the different isobutanol pathways could affect other aspects of the strain’s proteome. Pathway enrichment analysis using Funspec [48] was performed on the statistically significant protein hits from the cIBA[8568] and cIBA[LV5] strains to identify any enriched patterns. Proteins at a higher abundance compared to WT were enriched (P < 0.05) as indicated by the presence of a number in the heatmap cell. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.) clusters, inducing a conformational change that activates the transcription of genes to help regulate iron storage including CCCI, TWY1, GRX4, and CUP1 (Fig. 5a). Indeed, protein levels of Ccc1p, Tyw1p, and Grx4p were elevated in the cIBA[8568] and cIBA[LV5] strains for at least one of the time points with Tyw1p having the highest fold change of 1.7 at10 h when compared to WT (Fig. 5b). This increase in Yap5p responsive genes indicated there was an increased number of 2Fe–2S clusters in the cytosol of the cIBA[8568] and cIBA[LV5] strains compared to WT, which was expected with the increased level of Atm1p.

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hypothesize the fra2 deletion had no effect on isobutanol titer in the mIBA^{ILV5} strain because the cofactor availability for the mitochondrial-localized Ilv3p was not limited.

We next tested if the deletion of FRA2 specifically benefited Ilv3p by looking at the intracellular metabolite levels of the enzyme’s reactant, DHIV, and product, KIV (Fig. 6c). Indeed, the cIBA^{ILV5} fra2Δ strain had lower levels of DHIV compared to the cIBA^{ILV5} strain indicating the Ilv3p bottleneck was partially relieved; the normalized peak area (peak area/OD<sub>600</sub>) of DHIV was 1.7-fold (<i>P</i> < 0.05) and 2.9-fold (<i>P</i> < 0.005) lower in cIBA^{ILV5} fra2Δ compared to cIBA^{ILV5} at 10 and 26 h, respectively. The KIV level was also measured, but we did not observe an increased level of KIV in the cIBA^{ILV5} fra2Δ strain compared to the cIBA^{ILV5} strain as one might expect from relieving the bottleneck at Ilv3p. Instead, the KIV level between the engineered strains was not significantly altered, except at 26 h where the cIBA^{ILV5} strain had a 3.5-fold increase in KIV compared to the cIBA^{ILV5} fra2Δ strain (<i>P</i> < 0.05). We suspect an accumulation of KIV was not seen because KIV is readily consumed in the subsequent steps. Taken together, the fra2 mutation in the cIBA^{ILV5} strain resulted in an increase in isobutanol titer, potentially by partially overcoming the 2Fe–2S cluster cofactor limitation for cytosolic Ilv3p.

4. Conclusions

Here, we combined transcriptomic, proteomic, and metabolomic analyses to generate a wealth of data on how enzyme pathway localization and redox cofactor-balancing affect isobutanol biosynthesis and physiology in genetically engineered <i>S. cerevisiae</i>. Localizing isobutanol pathway enzymes to the mitochondria resulted in higher titers, while cytosolic localization resulted in much lower titers. Metabolomic analysis uncovered a potential limitation in cytosolic Fe–S cluster biogenesis. This limitation could be overcome by increasing Fe uptake through deletion of FRA2. This strategy of increasing the availability of iron in the cell was also successful in increasing the activity of another Fe–S cluster requiring enzyme xylonate dehydratase; however, this strategy may not be effective for all Fe–S cluster requiring enzymes as this strategy had no effect on 6-phosphogluconate dehydratase activity [<i>A</i>]. While we did not observe a beneficial effect in having a redox cofactor-balanced isobutanol pathway, we hypothesize that the cofactor imbalance will have to be resolved for the capacity of the isobutanol pathway to be enhanced. Furthermore, any problems caused by an imbalance will be exacerbated when ethanol flux is displaced to enhance the rate of isobutanol synthesis. Future functional genomics studies with engineered strains disabled for ethanol production should uncover novel genetic targets for achieving greater isobutanol titers and yields.

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**Data availability**

The mass spectrometry proteomics data have been deposited to the MassIVE database with the identifier MSV000088169. The transcriptomics data discussed in this publication has been deposited in...
NCBI’s Gene Expression Omnibus [59] and is accessible through GEO Series accession number GSE186126 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE186126). Intracloudal metabolomics data is available on GitHub (https://github.com/AmadorNoguezLab/compa rtementalized-isobutanol-pathways-in-S.-cerevisiae). The full isobutanol pathway cassette sequence containing mitochondrial-localized enzymes and flanking HO arms is available on GenBank with the identifier M2541859 (Materials and methods 2.2).

Credit authorship statement

Funding acquisition: CTH, TKS, APG, DAN, YZ, BFP, JJC Conceptualization: CTH, TKS, APG, DAN, YZ, BFP, TJB, ERW, RLW, and FVG

Strain engineering: TKS, MT, FVG, LL, MJ, MVS Design of experiments: FVG, TKS, BFP, CTH

Investigation: TKS, MT, FVG, JJB, JFW, TJB, ERW, JJD, DX, JS, and SG

Data analysis: TJB, ERW, LKM, FVG, MAM

Data visualization: FVG, ERW, LKM

Formal analysis: FVG, ERW

Writing – original draft: FVG

Writing – methods: TKS, ERW, LKM, FAM, JFW

Writing – review & editing: BFP, TKS, APG, CTH, TJB, ERW, FVG, JJB

Declaration of competing interests

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjynbio.2022.02.007.

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