Title Page

Engineering a functional 1-deoxy-D-xylulose 5-phosphate (DXP) pathway in *Saccharomyces cerevisiae*

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Abstract

Isoprenoids are used in many commercial applications and much work has gone into engineering microbial hosts for their production. Isoprenoids are produced either from acetyl-CoA via the mevalonate pathway or from pyruvate and glyceraldehyde 3-phosphate via the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway. *Saccharomyces cerevisiae* exclusively utilizes the mevalonate pathway to synthesize native isoprenoids and in fact the alternative DXP pathway has never been found or successfully reconstructed in the eukaryotic cytosol. There are, however, several advantages to isoprenoid synthesis via the DXP pathway, such as a higher theoretical yield, and it has long been a goal to transplant the pathway into yeast. In this work, we investigate and address barriers to DXP pathway functionality in *S. cerevisiae* using a combination of synthetic biology, biochemistry and metabolomics. We report, for the first time, functional expression of the DXP pathway in *S. cerevisiae*. Under low aeration conditions, an engineered strain relying solely on the DXP pathway for isoprenoid biosynthesis achieved an endpoint biomass 80% of that of the same strain using the mevalonate pathway.

Keywords: MEP pathway; yeast; metabolic engineering; terpene.

1. Introduction

Isoprenoids, constituting one of the largest and most diverse groups of natural products, are made by all free-living organisms and range from essential metabolites such as sterols and quinones to more specialized compounds such as pinene and rubber (1). Nature employs two

routes for the biosynthesis of isoprenoids: the mevalonate and 1-deoxy-D-xylulose 5-phosphate (DXP) pathways (the latter also known as the methylerythritol 4-phosphate, or MEP, pathway) (2-4). Although plants use both pathways (synthesizing mevalonate in the cytosol and DXP in the plastid) most other organisms utilize only one of them, with the DXP route dominant in bacteria and the mevalonate route in eukaryotes and archaea (2, 5, 6). Some archaea also use a modified version of the mevalonate pathway (7). Both pathways produce a mixture of the C5 compounds isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the building blocks for isoprenoid biosynthesis, in seven steps starting from central metabolism. Mevalonate is generated from three molecules of acetyl-CoA, while DXP is synthesized by condensation of the glycolysis intermediates glyceraldehyde 3-phosphate (G3P) and pyruvate (Fig. 1).

Saccharomyces cerevisiae, like other non-plastid-bearing eukaryotes, exclusively uses the mevalonate pathway for isoprenoid biosynthesis.

There are several benefits to using the DXP pathway in *S. cerevisiae* for isoprenoid production. Some of the most successful strategies for engineering increased isoprenoid production have involved expressing non-native genes or entire pathways in order to circumvent native regulatory mechanisms and competition for intermediates. Examples include expression of the entire mevalonate pathway in *Escherichia coli* (8) and the production of sesquiterpenes in the plant plastid (9). There are also specific advantages to using the DXP pathway instead of the native mevalonate pathway for producing isoprenoid-based chemicals and fuels in yeast. First, a higher maximum stoichiometric yield is expected from the DXP pathway (0.30 g/g for production of a terpene olefin such as isoprene or farnesene from glucose) than the native

mevalonate pathway (0.25 g/g) (10). Second, the DXP pathway requires approximately half the amount of oxygen than the mevalonate pathway (11).

Several attempts to functionally express the DXP pathway in *S. cerevisiae* have been reported previously. In the first report, seven genes from *E. coli* encoding the core DXP pathway enzymes were expressed in *S. cerevisiae* and the pathway was deemed to be functional based on growth of this strain in the presence of lovastatin, an inhibitor of the mevalonate pathway enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (12). However, a follow-up study by the same group demonstrated that insensitivity to lovastatin was insufficient evidence for DXP pathway functionality since it was not possible to complement a knockout of the mevalonate pathway gene *ERG13*, encoding HMG-CoA synthase (13). Soon afterwards, an independent study confirmed that expression of the *E. coli* DXP pathway genes was insufficient to complement an *ERG13* knockout in *S. cerevisiae* (14). Both groups came to the conclusion that flux through the pathway was arrested at 2C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP), the fifth pathway intermediate and substrate for IspG, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate (HMBPP) synthase (Fig. 1). This is not entirely surprising since IspG is considered to be the slowest enzyme in the DXP pathway (15-17), and MEcPP is often found to accumulate in organisms that utilize the DXP pathway (18-20). Most likely, a key obstacle to functional expression of IspG in yeast is the complexity of the reaction, involving an iron-sulfur cluster and accessory reductases (Fig. 2).

IspG and IspH, which catalyze the last two steps of the DXP pathway, each mediate 2-electron reduction reactions via endogenous [4Fe-4S] clusters coupled with accessory redox enzymes

and NADPH (21-23). In *E. coli* the redox partners are flavodoxin and flavodoxin reductase, encoded by the genes *fldA* and *fpr*, respectively; *fldA* is essential to *E. coli* for this purpose (24). In the case of plant plastids and cyanobacteria, where flavodoxins are normally absent, HMBPP synthase relies on other reducing partners, for example GcpE in *Thermosynechococcus elongatus* relies on ferredoxin (25), while GcpE in *Arabidopsis thaliana* can use ferredoxin but can also draw electrons directly from photosynthetic thylakoid membranes (26). *S. cerevisiae* harbors mitochondrial ferredoxin and ferredoxin reductase but lacks the ability to generate cytosolic [2Fe-2S] clusters, a requirement for plant-type ferredoxins (27). Since yeast, like other non-photosynthetic eukaryotes, lacks flavodoxin it remains that the supply of an exogenous flavodoxin and reductase is a necessary prerequisite for constructing a functional DXP pathway in the *S. cerevisiae* cytosol (28).

The next complicating factor for functional IspG expression in yeast is the assembly of its essential [4Fe-4S] cluster. All bacteria use either the ISC (iron-sulfur cluster) or SUF (sulfur mobilization) machinery for assembly of [4Fe-4S] clusters for integration into apo-IspG, *E. coli* relying primarily on the former, encoded by the *isc* operon (29). Likewise, the [4Fe-4S] cluster for GcpE in the plant plastid is assembled by prokaryotic-type machinery, analogous to the bacterial SUF system (30). However, *S. cerevisiae* assembles all of its [4Fe-4S] clusters in mitochondria, using machinery analogous to the bacterial ISC system, and then exports these clusters to the cytosol where they are integrated into apo-proteins by the Cytosolic Iron-Sulfur Assembly (CIA) machinery (30, 31). Yeast uses the CIA machinery to integrate [4Fe-4S] clusters into native cytosolic enzymes (such as LEU1, isopropylmalate isomerase) but many of the details, such as the exact mechanism of export from mitochondria, are not yet fully understood

(30, 32). Furthermore, in *E. coli* an A-type carrier protein was discovered to be essential for efficient [4Fe-4S] cluster insertion into apo-IspG. The encoding gene, *erpA*, is essential for respiratory growth in *E. coli* (33) but is not present in *S. cerevisiae*.

Despite co-expression of *erpA* and the redox partners, *fldA* and *fpr*, Partow et al. found the *E. coli* IspG to be non-functional in *S. cerevisiae* (13). Faced with a similar result, having expressed these three genes together with the entire ISC machinery from *E. coli*, Carlsen et al. decided to investigate whether an alternative bacterial [4Fe-4S] enzyme could be functionally expressed in the yeast cytosol (14). They successfully complemented a *leu1* auxotrophy by expression of the orthologous isopropylmalate isomerase from *E. coli*, a heterodimeric [4Fe-4S] cluster protein. On the other hand, a recent attempt to replace glycolysis in *S. cerevisiae* with the bacterial Entner-Doudoroff pathway was unsuccessful and this was attributed to the lack of functional [4Fe-4S] cluster assembly into 6-phosphogluconate dehydratase (34). It therefore seems apparent that assembly of [4Fe-4S] clusters into heterologous proteins in the yeast cytosol is possible but is not always successful.

Here we report the functional expression of the DXP pathway in *S. cerevisiae*. To achieve this, a number of orthologous genes encoding IspG, IspH, flavodoxin (Fld), and flavodoxin/ferredoxin NADP⁺-reductase (FNR) were selected from a wide range of species and screened for solubility and activity in yeast. Flux through the DXP pathway, demonstrated by ¹³C-feeding studies in a mevalonate-auxotrophic strain of *S. cerevisiae*, was improved to the point where the major pathway bottleneck was no longer IspG but rather IspD. Resolution of the IspD bottleneck

resulted in a strain capable of growth in the absence of mevalonate, relying solely on the transplanted DXP pathway.

2. Materials and methods

2.1 Deletion of ERG13 and improvement of growth rate on mevalonate

Unless otherwise stated, yeast was grown at 30 °C in synthetic defined (SD) medium, consisting of 5.7 g/L Difco yeast nitrogen base (Becton, Dickinson & Co.), a carbon source, and the manufacturer-recommended amount of Complete Supplemental Mixture (Sunrise Science) lacking certain amino acids where appropriate for selection. An *ERG13* knockout cassette consisting of a *LEU2* marker cassette flanked by sequences matching the 5' and 3' regions of the *ERG13* coding sequence was assembled using splicing by overlap extension (SOE) PCR and used to transform *S. cerevisiae* CEN.PK2 to generate strain Y5221. Colonies emerged after 7 days growth at 30 °C on SD-leu (SD medium lacking leucine) supplemented with 5 g/L mevalonate and successful deletion of *ERG13* was confirmed by PCR. Additional details including oligonucleotide primer sequences are provided in the Supplementary Material.

To select for spontaneous mutants with improved growth rates, Y5221 cells were grown on SD + 5 g/L mevalonate, washed, and spread at a density of 5×10^6 cells per plate onto SD agar containing 2 g/L mevalonate. After 5 days of growth at 30°C, single colonies were visible and the three fastest growing mutants were selected (Y5822, Y5823, Y5824) for genome sequencing and growth rate determination (See the Supplementary Material and Supplementary Table S2). Strain Y5823 (*erg13::LEU2*, *PRM10* (L156Q)) was selected for further use, based on a doubling time similar to that of the parent CEN.PK2 in SD medium supplemented with 5 g/L mevalonate.

2.2 Construction of the top portion of the DXP pathway

Sequences encoding the first five DXP pathway enzymes (Dxs, IspC, IspD, IspE, and IspF) from *E. coli* MG1655 were codon optimized for expression in yeast and synthesized along with contiguous yeast terminators (Integrated DNA Technologies, Inc.). SOE PCR was used to join each of these parts with promoters amplified from *S. cerevisiae* CEN.PK2 (*TDH3*, *TEF2*, *TDH2*, *TDH3*, and *TEF2*, respectively) and assembled into two larger fragments, each containing sequence homologous to the *S. cerevisiae* *HO* (YDL227C) gene at one end and an overlapping fragment of a *URA3* marker cassette at the other. These two parts were transformed into Y5823, and following selection on SD-ura agar, the 5 DXP expression cassettes were confirmed to have integrated into the *HO* locus, generating strain Y6080. To increase carbon flux to MEcPP, an additional copy of the P_{TEF2} -*ispC* cassette was integrated into the *LEU2* locus of Y6080 using a *HIS3* marker, generating the strain Y6081. Further cloning details are provided in the Supplementary Material.

2.3 Purification of IspG and IspH proteins from *S. cerevisiae*

To enable purification of IspGs and IspHs from *S. cerevisiae*, each gene was fused to DNA encoding an N-terminal Strep-tag II (35) and expressed from the *GAL1* promoter on plasmid pRS426 (36). Proteins were expressed in strain BCY123, deficient in three proteases (*YPL154C*, *YEL060C*, and *YIL015W*) and harboring an extra chromosomal copy of *GAL4*, driven by the *GAL1* promoter (37). Conditions for purification and detection of the tagged proteins are provided in the Supplementary Material.

2.4 In vitro assay of IspG and IspH purified from *S. cerevisiae*

All work was performed in an anaerobic chamber at ambient temperature using degassed buffers. For assays with artificial electron donors, each 50 μ L reaction contained 100 mM Tris (pH 8.0), 5 mM sodium dithionite, 1 mM methyl viologen, 5 μ L of purified enzyme, and 320 μ M MEcPP or 380 μ M HMBPP as appropriate. At the desired time points, 10 μ L of the assay was added to 490 μ L methanol containing 1 μ g/mL of the internal standard farnesyl thiolodiphosphate (TFPP, Echelon Biosciences, Inc.). Samples were then stored at -80 °C until ready for analysis by liquid chromatography-mass spectrometry (LC-MS). IspG and IspH activities were measured by the rates of product formation (HMBPP and IPP/DMAPP, respectively). IspG and IspH enzymes were also assayed in combination with various Flds and FNRs that were purified from *S. cerevisiae*. The procedure was the same as above except that each reaction contained 100 mM Tris buffer (pH 8.0), 5 μ L each of freshly purified FNR, Fld, IspG or IspH, and 320 μ M MEcPP or 380 μ M HMBPP as appropriate.

2.5 ⁵⁵Fe incorporation assay

Iron incorporation into IspG and IspH proteins expressed in *S. cerevisiae* was measured using a method adapted from Pierik et al. (38). Briefly, *S. cerevisiae* BCY123 harboring a plasmid encoding a Strep-tagged IspG or IspH protein (described above) was grown for 20-24 h in the presence of ⁵⁵FeCl₃. Following purification of the Strep-tagged protein, radioactivity was measured on a Beckman Coulter LS 6500 scintillation counter and normalized to the protein concentration. The endogenous yeast cytosolic [4Fe4S] protein, LEU1, was expressed and purified under the same conditions to serve as a benchmark. Further details are provided in the Supplementary Material.

2.6 Quantification of DXP pathway intermediates

Yeast cells from 5 mL of culture were pelleted by brief centrifugation (3,000 x g for 3 min) and lysed by the addition of 1 mL ice-cold 50% (vol/vol) methanol (in water) to the cell pellet and bead beating in the presence of 0.5 mm glass beads. Following centrifugation (15,000 x g for 10 min at 4°C), the lysate was clarified by passing through a 3,000-MW-cutoff (MWCO) Amicon Ultra 0.5-ml filter (Millipore). Analysis was performed by LC-MS using authentic standards (Echelon Biosciences, Inc.) as described previously (20).

2.7 Construction of full DXP strains / Incorporation of *ispG*, *ispH*, *fld* and *FNR* into the DXP-TOP strain

Strain Y6081, harboring an *ERG13* deletion and the top portion of the DXP pathway, served as the parent for expression of the lower portion of the DXP pathway (*ispG*, *ispH* and associated genes). The *FNR* gene, *AtrFNR* (*A. thaliana* root), was integrated into the genome in all cases, while various combinations of *ispG*, *ispH* and *Fld* genes were expressed either individually or as fusion proteins from cassettes integrated into the genome or maintained on 2-micron plasmids. Further details are provided in the Supplementary Material.

2.8 Measurement of flux to ergosterol via the DXP pathway in *S. cerevisiae*

Yeast strains harboring the DXP pathway were cultured overnight on SD medium containing 2% (w/v) glucose and 15 mM mevalonate and then inoculated into fresh media containing 0.5% ¹³C₆-galactose (fully-labeled) and 15 mM mevalonate (unlabeled). Ergosterol was extracted from cultures and analyzed by GC-MS as described previously (39) but the mass spectrometer was set to detect ions between m/z 396 (the ergosterol parent ion, unlabeled) and m/z 424

(fully ^{13}C -labeled ergosterol). The proportion of ergosterol derived from the DXP pathway was calculated by integration of extracted ion chromatograms corresponding to ^{13}C -labeling of the ergosterol parent ion (ions between m/z 399 and 424). Equivalent cultures supplied with 0.5% galactose (unlabeled) as well as parent strain (Y6084) cultures supplied with 0.5% $^{13}\text{C}_6$ -galactose were included to establish baselines.

*2.9 Growth of *S. cerevisiae* in the absence of the mevalonate pathway*

Strains were cultured overnight to similar ODs in SD medium (SD-ADE for the strains harboring plasmids) containing 0.3% glucose, 0.5% galactose, and 15 mM mevalonate. Cells were then centrifuged briefly to remove media, washed and then resuspended in media containing no mevalonate, and ODs were again measured. These cells were then inoculated into 30 mL SD (or SD-ADE) medium containing 0.5% galactose to an OD of 0.03, and divided between six culture tubes. The media in three of the tubes was supplemented with 15 mM mevalonate, and all tubes were incubated at 30 °C with shaking at 50 rpm.

3. Results

*3.1 Knockout of the mevalonate pathway in *S. cerevisiae* CEN.PK2*

Screening for DXP pathway functionality via resistance to the HMGR inhibitor lovastatin was deemed to be unreliable and subject to false positives, likely due to acquired resistance, as reported previously (13). To provide a definitive metric for DXP functionality, deletion of one of the first three steps in the mevalonate pathway in *S. cerevisiae* was considered the best approach since it enables screening for mevalonate-independent growth. It also allows for the

quantitative measurement of DXP pathway flux by feeding with a fully labeled carbon source in combination with unlabeled mevalonate. Based on the observed low rate of spontaneous suppressor mutations ($< 10^8$), *ERG13* was selected for deletion from *S. cerevisiae* CEN.PK2, generating strain Y5221. This strain exhibited a slow growth rate and low transformation efficiency even when fed mevalonate at concentrations of 5 g/L. Following selection in synthetic defined (SD) medium supplemented with 2 g/L mevalonate, a spontaneous mutant (Y5823) was identified that exhibited a growth rate similar to that of its parent, CEN.PK2. Genome sequencing of Y5823 revealed the acquisition of a point mutation in *PRM10*. *PRM10* is predicted to be a pheromone-regulated membrane protein whose function is not yet known (see the Supplementary Material for further details). Since the low growth rate of Y5221 is likely caused by the limitation in mevalonate uptake (Table S2), the PRM10 mutation presumably increases the uptake rate of mevalonate from the medium.

3.2 Expression of the entire DXP pathway from E. coli in S. cerevisiae

Initial attempts to express the DXP pathway in *S. cerevisiae*, using genes sourced from *E. coli*, yielded results in agreement with findings from other groups (12-14). Expression of the seven core pathway enzymes and associated redox partners, *fldA* and *fpr*, in several conformations (under control of *GAL1* and *GAL10* promoters on low copy and high copy plasmids or under strong constitutive promoters and integrated into the chromosome) yielded no evidence of flux through the DXP pathway. The metabolite MEcPP was found to accumulate in all cases, implicating IspG as the pathway bottleneck.

3.3 Functional expression of top portion of the DXP pathway

The first 5 genes of the *E. coli* DXP pathway (*dxs*, *ispC*, *ispD*, *ispE*, and *ispF*), codon optimized and placed under control of strong constitutive yeast promoters, were integrated at a single locus in Y5823 to create strain Y6080 (Fig. 3). The function of the top part of the pathway was confirmed by the detection of MEcPP at intracellular levels of 300-400 μ M. Analysis of DXP pathway intermediates in Y6080 revealed accumulation of DXP at intracellular levels of around 100 μ M, suggesting that DXP reductoisomerase (Dxr, encoded by *ispC*) is limiting. An additional copy of the *ispC* cassette was introduced to further optimize the upper pathway flux, generating strain Y6081. The accumulation MEcPP to intracellular concentrations in the range of 700-900 μ M was observed in Y6081 (Supplementary Fig. S1).

3.4 Investigation of *IspG* solubility in *S. cerevisiae*

To investigate the functionality of *IspG* from *E. coli* (EclspG) as expressed in the yeast cytosol, *ispG* harboring an N-terminal Strep-tag II was expressed from the *GAL1* promoter on the 2-micron plasmid pRS426 (36) in *S. cerevisiae* strain BCY123 (37). BCY123 was designed to facilitate strong protein expression by deletion of three proteases (*YPL154C*, *YEL060C*, and *YIL015W*) and insertion of an extra chromosomal copy of *GAL4*, driven by the *GAL1* promoter. *IspG* was expressed under normal growth conditions (shaking at 200 rpm) and then purified anaerobically under either native or denaturing conditions. Protein solubility was assessed by comparing the *IspG* concentration in the two protein extracts following western blotting using a primary antibody for the Strep-tag II. The concentration of *IspG* in the extract processed under denaturing conditions was much higher than that from the native extract indicating that the majority of the protein expressed in yeast was in an insoluble form.

To determine whether IspG homologs from other host organisms may be more soluble than EclspG when expressed in the yeast cytosol, we used the approach above as a screen for solubility (Supplementary Fig. S2). An additional 44 IspG homologues (Supplementary Fig. S3) were sourced from a diverse group of organisms (including bacteria, protozoa, algae, and plants), codon optimized for *S. cerevisiae*, expressed and purified under the same conditions described for EclspG. A total of 29 IspG homologues out of 44 were soluble in the yeast cytosol to an extent greater than EclspG, as judged by western blotting method above (Supplementary Table S11).

3.5 *In vitro* activity of IspGs purified from *S. cerevisiae*

The 27 most soluble IspG orthologs, purified from yeast, were next tested for *in vitro* conversion of MEcPP to HMBPP in the presence of artificial electron donors, methyl viologen and dithionite. The majority of the IspG orthologs, including several that were highly soluble when expressed in the yeast cytosol (for example, GcpE from *A. thaliana*, AtIspG) displayed little or no activity under these conditions. The highest specific activities in the presence of artificial electron donors (ranging from 1 to 7 pmol/min/pmol enzyme) were encountered in ten IspG orthologs from various sources including two *Bacillus* species (*Bacillus subtilis*, BsIspG, and *Bacillus thuringiensis*, BtIspG), a unicellular alga (*Ostreococcus tauri*, OtIspG) and a higher plant (*Vitis vinifera*, VvIspG) (Supplementary Table S11).

As a point of comparison, we overexpressed the native EclspG in *E. coli* and determined the specific activity under the same conditions as the enzymes purified from yeast. Previous work demonstrated that the specific activities of IspH and IspG overexpressed in *E. coli* were

significantly higher when the ISC machinery was also overexpressed (21, 22). Therefore, *EcispG* was also overexpressed in a strain of *E. coli* harboring a plasmid bearing an additional copy of the *isc* operon (15). EclspG specific activity was over 20-fold higher as measured using the protein purified from *E. coli* overexpressing the *isc* operon, compared to protein purified from an equivalent strain containing native levels of ISC machinery (8.9 and 0.4 pmol/min/pmol enzyme, respectively). The specific activity for VvlspG purified from *S. cerevisiae* (6.3 pmol/min/pmol enzyme) was similar to that of EclspG purified from *E. coli* overexpressing the *isc* genes, indicating that, although specific activity levels are low, some of the lspG homologs expressed in yeast are as functional as the native EclspG enzyme, when expressed, purified, and assayed under similar conditions.

3.6 Screening for lspH functionality in *S. cerevisiae*

The same approach was used to screen lspH orthologs for functionality in yeast. A total of 17 lspH orthologs from diverse sources were expressed in BCY123 as Strep-tag II fusions, purified anaerobically, and analyzed by Western blotting for solubility as described above for lspG. Protein levels were generally higher for lspH than for lspG, such that the protein was visible in the Coomassie-stained gel prior to western blotting. Seven out of the 17 orthologs were soluble to some degree in yeast, and in vitro conversion of HMBPP to DMAPP in the presence of artificial electron donors was observed in four of the five enzymes tested, including lspH from *E. coli* (EclspH) (Supplementary Table S12). In fact, specific activity for EclspH purified from yeast (45 pmol/min/pmol enzyme) was 7-fold higher than the highest activity observed for an lspG (VvlspG) in the presence of artificial electron donors.

3.7 Screening for active redox partners in *S. cerevisiae*

Following the same approach as above, a variety of flavodoxins (Flds) and flavodoxin/ferredoxin NADP⁺-reductases (FNRs) were overexpressed and purified from *S. cerevisiae* (Supplementary Figs. S4 and S5). 13 out of 16 Flds and 10 out of 10 FNRs screened in yeast were found to be present mostly in soluble form. In order to identify the combinations of Flds and FNRs that afford the highest in vitro activity when combined with IspG or IspH, combinatorial assays were performed using several orthologs of each enzyme, all expressed in *S. cerevisiae* and purified anaerobically. It emerged from this initial screen that the FNRs in general were the most robust component of the enzyme triad, in that they were less sensitive to partner choice than the other components. Several FNRs, including AnaFNR (*Anabaena* PCC 7119), AtrFNR (*A. thaliana* root-localized), EcFNR (*E. coli*) and TeFNR (*Thermosynechococcus elongatus* BP-1), were found to be functional to a high degree with both IspG and IspH (Table S15). While BtIspG activity did not vary greatly when combined with these different FNRs, significantly higher activity was observed for EcIspH when combined with AtrFNR, and thus AtrFNR was selected as the best reductase.

The plant or algae-based IspGs that were quite active in the presence of artificial electron donors (e.g., VvIspG and OtIspG) were found to be inactive when tested in combination with the FNRs and Flds expressed here, possibly due to a preference for ferredoxin rather than flavodoxin partners. Since the [2Fe-2S] clusters required for plant-type ferredoxins are not available in the yeast cytosol, we focused on screening for Fld partners that worked best with the remaining bacterial IspGs (BtIspG, BsIspG, BcIspG, from *Bacillus coagulans*, and TtIspG, from *Thermus*

thermophilus). Using AtrFNR as the reductase partner, in vitro assays were carried out with the 13 Flds found to be soluble in yeast. Choice of Fld appears to be a critical factor for achieving IspG functionality in vitro, BsFld being the most active with all of the IspGs tested (Supplementary Fig. S6). BtIspG was most active of the four IspGs when combined with BsFld, although its specific activity was 3-fold lower than when combined with artificial electron donors (Supplementary Table S11). Screening of four IspHs in combination with either EcFld or BsFld demonstrated that both Flds were active with all four IspHs, the combination of EcIspH and EcFld being the most active (Supplementary Fig. S7). The specific activity of EcIspH when combined with EcFld was 18-fold lower than activity in the presence of artificial electron donors, but still almost 5-fold higher than the best IspG-Fld combination.

3.8 Overexpression of ISC machinery in *S. cerevisiae*

A ^{55}Fe incorporation assay was used to investigate whether cytosolic overexpression of the ISC genes from *E. coli* would have any influence on [4Fe-4S] cluster assembly into IspG and IspH. Eight expression cassettes, harboring seven genes from the *E. coli* *isc* operon (*hscA*, *iscA*, *cyaY*, *iscS*, *iscU*, *hscB*, and *fdx*) as well as *erpA* (33, 40), under control of constitutive promoters were chromosomally integrated into strain BCY123 harboring either IspG or IspH genes on plasmids (see the Supplementary Material for further details). The IspG and IspH genes were from both *E. coli* and *Thermosynechococcus elongatus* and contained N-terminal Strep-tags to enable purification. For comparison, a Strep-tagged version of the native yeast cytosolic [4Fe-4S] protein 3-isopropylmalate dehydratase (LEU1) was also expressed in BCY123 on a plasmid. Following growth of cultures supplemented with ^{55}Fe , the IspG and IspH proteins were purified

under anaerobic conditions alongside LEU1. ^{55}Fe was incorporated into EclspH at levels similar to that of LEU1, while ^{55}Fe incorporation into EclspG was negligible, consistent with the poor solubility and lack of activity detected for EclspG expressed in yeast. Although all eight of the ISC proteins were detected in cytosolic extracts, their presence had no significant effect on ^{55}Fe incorporation into the lspG/H proteins (Supplementary Fig. S8).

3.9 Fusion of flavodoxin to lspG and lspH

Fusion proteins were constructed for EclspH and three lspG orthologs (BtlspG, BslspG and TtlspG) to EcFld and BsFld, respectively. Measurement of in vitro activity demonstrated that the lspG enzymes were only active with C-terminal Fld fusions, while lspH was only active with an N-terminal Fld. Although in vitro activity showed no advantage to using fusions over individual enzymes (data not shown) it was considered a possibility that the stoichiometric balance of a fusion protein may have advantages in vivo.

3.10 Demonstration of flux via the DXP pathway in *S. cerevisiae*

The screening approaches described above suggests that the most active enzymes among those we expressed in yeast are AtrFNR; BtlspG or BslspG coupled with BsFld; and EclspH coupled with either EcFld or BsFld. Various permutations of these genes were introduced into the mevalonate-auxotrophic strain containing the top portion of the DXP pathway, Y6081, to complete the pathway. DXP pathway throughput was quantified by feeding with fully ^{13}C -labeled galactose coupled with unlabeled mevalonate and measuring the extent of ^{13}C -labeling in ergosterol by gas chromatography-mass spectrometry (GC-MS) (Fig. 4). A chromosomally-integrated $P_{\text{GAL10}}\text{-AtrFNR}$ cassette was present in all strains tested, with the focus on optimizing

expression of IspG, IspH, and Fld. Testing fusion proteins initially, a small but measurable flux through the DXP pathway (approximately 1.35 % of ergosterol was ^{13}C -labeled) was observed in strain Y6544, harboring a chromosomally-integrated $P_{\text{GAL1}}\text{-EcFld-EcispH}$ cassette and a 2-micron plasmid bearing $P_{\text{GAL1}}\text{-BtispG-BsFld}$ (Table 1). Flux was improved slightly (resulting in 1.62% ^{13}C -labeling of ergosterol) when the $P_{\text{GAL1}}\text{-BtispG-BsFld}$ was instead chromosomally integrated. Expression of IspG, IspH and Fld as separate proteins improved pathway throughput significantly, exceeding 5% ^{13}C -labeling of ergosterol in strain Y6084/pAM2397, which contains chromosomally-integrated $P_{\text{GAL1}}\text{-EcispH}$ and $P_{\text{GAL1}}\text{-BsFld}$ cassettes and the 2-micron plasmid pAM2397 harboring a $P_{\text{GAL1}}\text{-BtispG}$ cassette. Substitution of *BtispG* for *BsispG* (strain Y6084/pAM2398) resulted in a further small increase in pathway flux.

3.11 Effect of oxygen on DXP pathway flux

Since IspG is known to be sensitive to oxidative stress (18, 41) we investigated whether flux via the DXP pathway would be higher when oxygen was limited. Strain Y6084/pAM2398 was cultured under conditions of reduced aeration by decreasing the culture shaking speed. Compared to standard conditions (shaking at 200 rpm), ^{13}C -labeling of ergosterol doubled in cultures shaken at 100 rpm and increased almost 5-fold to 24.5% when shaken at 50 rpm (Fig. 5A).

3.12 Metabolite-guided DXP pathway balancing

MEcPP was observed to accumulate in all of the strains we examined, inviting the assumption that IspG remained the primary pathway bottleneck. To determine if IspG was indeed still the limiting enzyme under conditions of low aeration, DXP pathway intermediates were quantified

in cultures exhibiting various levels of DXP pathway flux (Fig. 5B). Under conditions of normal aeration, when pAM2398 (BtlspG) is added to the strain Y6084 to complete the DXP pathway, DXP metabolite levels fall to almost zero (Y6084-a, Fig. 5B), indicating that Dxr is not limiting. As aeration is decreased (by reducing the shaking speed to 100 rpm) DXP increases again to about 50% of Y6084 levels, but this may be due to a decreased availability of NADPH, required for Dxr-mediated reduction of DXP, rather than an enzyme limitation. Not surprisingly, when IspG is added to Y6084, MEcPP levels also drop significantly since the pathway has been completed. A significant 3-fold increase in MEP concentration occurred when the shaking speed was reduced from 200 to 100 rpm, indicating that IspD may have become a significant pathway bottleneck. To test this, an additional copy of *ispD* was added to the pAM2398 plasmid, under control of the native *BIO2* promoter (Y6084-b, Fig. 5) and DXP pathway flux increased to the point where 58% of ergosterol was ¹³C-labeled. In response to the increased expression of IspD, MEP and DXP levels decreased while CDP-ME and MEcPP levels increased slightly.

3.13 Growth of S. cerevisiae in the absence of the mevalonate pathway

Strains Y6084, Y6084/pAM2398, and Y6084/pAM2398-ispD were cultured under conditions similar to those used to test flux (in SD medium containing 0.5% galactose, shaken at 50 rpm) either with or without mevalonate. In the absence of mevalonate, Y6084 did not grow (as expected) and while the OD of Y6084/pAM2398 doubled in the first 24 hours, this strain also did not grow further beyond that point. However Y6084/pAM2398-ispD grew in the absence of mevalonate, reaching an OD around 80% of that of the same strain cultured in medium supplemented with 15 mM mevalonate (Fig. 6). The addition of the second copy of *ispD* to

Y6084/pAM2398 appears to have increased flux to the point where it is above the threshold to sustain isoprenoid synthesis sufficient for growth under low aeration conditions. The ability to grow this strain in the absence of mevalonate is expected to facilitate strategies for further improvements in DXP pathway functionality in yeast.

4. Discussion

Previous attempts to functionally express the DXP pathway in *S. cerevisiae* were unsuccessful in demonstrating a measurable level of flux through the pathway, but both correctly identified the sixth enzyme, IspG, as the primary issue (13, 14). Since IspG and IspH are both [4Fe-4S] enzymes, a natural starting point is to investigate whether the problem is predominantly a limitation in iron-sulfur cluster assembly. The importance of [4Fe-4S] cluster biosynthesis for these two enzymes was underlined by the recent demonstration that *E. coli* can survive a knockout of both Fe-S assembly systems, ISC and SUF, as long as mevalonate and the lower genes of the mevalonate pathway are provided to ensure a supply of IPP and DMAPP (42). It's also interesting to note that while pathogenic strains of *Listeria monocytogenes* maintain a full DXP pathway as well as a mevalonate pathway, its non-pathogenic relative *Listeria innocua* has specifically lost the genes encoding IspG and IspH, suggesting that these two [4Fe-4S] enzymes may constitute a high metabolic or physiological burden (43).

When it comes to expression of heterologous [4Fe-4S] proteins in the yeast cytosol, the likelihood of efficient Fe-S cluster biosynthesis is a largely unknown factor, since the CIA machinery is significantly different to the ISC and SUF systems that supply [4Fe-4S] clusters in bacteria and plastids. This unpredictability regarding the interchangeability of these systems is

exhibited by the fact that, in contrast to EclspG, EclspH was active when expressed in *S. cerevisiae*. ^{55}Fe was incorporated into EclspH at levels similar to that for the native yeast cytosolic [4Fe-4S] protein, LEU1, and expression of the ISC machinery in the yeast cytosol had no significant impact on ^{55}Fe incorporation into any of the lspG or lspH proteins tested. Therefore, considering that EclspG is not functional when expressed in the yeast cytosol and that reconstitution of the *E. coli* ISC machinery in this compartment is not trivial, it made sense to conduct a wide search for an lspG that is functional when expressed in yeast.

Several factors besides [4Fe-4S] cluster assembly came into play to accomplish the goal of functional lspG expression in yeast. The lspG screening process demonstrated that only two-thirds of the 45 lspG orthologs were soluble to a greater extent than the non-functional EclspG when purified from the yeast cytosol. It is likely that [4Fe-4S] cluster integration is a prerequisite for high protein solubility (44, 45), but solubility of lspG in yeast did not guarantee a functional enzyme. Even in the presence of artificial electron donors, several of the most soluble lspG proteins purified from yeast (e.g., AtlspG, FslspG from *Fibrobacter succinogenes*, and PblspG from *Prevotella buccae*) were not significantly active in vitro. Further screening by measurement of in vitro activity in the presence of flavodoxin partners narrowed the field to only four lspG orthologs, three from *Bacillus* spp. and one from *Thermus thermophilus* (all from the Terrabacteria group). Judging by the best enzyme/Fld combinations that emerged from our screening, BtlspG/BsFld (followed closely by BslspG/BsFld) and EcispH/EcFld, it seems that selection of enzyme and Fld from the same or closely-related species may be the best approach. There is evidence that the same general trend applies in selection of cytochrome P450s and their reductase partners (5).

4.1 Conclusion

Having demonstrated, for the first time, a measurable level of flux through the DXP pathway it became possible to make further improvements by exploring different gene combinations and expression levels. In balancing the pathway, examination of the relative change in concentration of DXP pathway intermediates in response to increasing flux levels (rather than absolute concentrations in a single strain) proved to be a useful approach, revealing the limitation in IspD activity. This also highlights the importance of regular checks on pathway metabolite concentrations over the course of a metabolic engineering project. It also became apparent that aeration had a significant impact on pathway flux. In plants it has become clear that IspG (GcpE) is oxygen-sensitive and that repair mechanisms play a large role in keeping the DXP pathway functional under conditions of oxidative stress (18, 46). Improvement of DXP pathway flux by limiting oxidative stress to IspG may have tradeoffs in other aspects of the pathway; the increase in DXP and MEcPP metabolite levels observed when aeration was reduced (Fig. 5B) may be due in part to a limitation in NADPH.

The DXP pathway became functional to the point where it could sustain moderate growth of *S. cerevisiae* in the absence of the mevalonate pathway but we were unable to achieve growth on agar in the absence of mevalonate. Even growth in liquid medium was only possible under the condition of low aeration, highlighting the need to address the IspG oxygen sensitivity issue. There is also possibly an issue of yeast lacking the machinery to repair damaged IspG [4Fe-4S] clusters, which would result in the DXP pathway becoming increasingly dysfunctional. Another possibility, albeit more remote, is that mevalonate, mevalonate 5-phosphate, or mevalonate

pyrophosphate are required for viability via some unknown mechanism. A third option is that it is difficult to find aeration conditions that accommodate both the NADPH requirement for the DXP pathway, and the oxygen-sensitivity of IspG. Future progress may come from a deeper understanding of [4Fe-4S] cluster biosynthesis in the yeast cytosol and cluster assembly and repair mechanisms for IspG.

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Figure Legends

Fig. 1. Two different pathways exist for the biosynthesis of isoprenoid precursors (IPP and DMAPP). (A) The mevalonate pathway (MVA pathway) in animals, plants (cytosol), fungi, and archaea. AACT (acetoacetyl-CoA thiolase), HMGS (3-Hydroxy-3-methyl-glutaryl-CoA synthase), HMGR (3-hydroxy-3-methyl-glutaryl-CoA reductase), MVK (mevalonate kinase), PMK (phosphomevalonate kinase), MPD (mevalonate diphosphate decarboxylase), IDI (isopentenyl diphosphate:dimethylallyl diphosphate isomerase). AcCoA (acetyl-CoA), AcAcCoA (acetoacetyl-CoA), Mev-P (mevalonate phosphate), Mev-PP (mevalonate diphosphate), IPP (isopentenyl diphosphate), DMAPP (dimethylallyl diphosphate). (B) The 1-deoxy-D-xylulose 5-phosphate pathway (DXP pathway) in eubacteria, green algae, and the plastids of higher plants. DXS (1-deoxy-D-xylulose 5-phosphate synthase), IspC (1-deoxy-D-xylulose 5-phosphate reductoisomerase), IspD (2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase), IspE (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), IspF (2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), IspG (2-C-methyl-D-erythritol-2,4-cyclodiphosphate reductase), IspH (4-hydroxyl-3-methylbut-2-enyl diphosphate reductase), Fld (flavodoxin), FNR (flavodoxin reductase).

Fig. 2. IspG-catalyzed reaction depends on a functional [4Fe-4S] cluster and redox partner proteins for electron transfer. The conversion of MEcPP to HMBPP requires two electrons (2e) that can be supplied by NADPH via a pair of redox proteins flavodoxin (or ferredoxin) and flavodoxin reductase. The iron-sulfur cluster of IspG uses these two electrons to reduce the substrate to produce HMBPP. For in vitro, an artificial electron donor can be used to replace the NADPH/FNR/Fld redox system to supply the required two electrons. IspH-catalyzed conversion of HMBPP to IPP and DMAPP involves a similar redox system to provide two electrons to its [4Fe-4S] cluster for catalysis.

Fig. 3. Schematic of selected strains used in this study. Promoters, coding sequences, and terminators are depicted as green arrows, blue boxes, and red boxes, respectively. All genes encode proteins from *E. coli* MG1655 unless indicated (Bs, *B. subtilis*; Atr, *A. thaliana* root; Bt, *B. thuringiensis*). All cassettes were chromosomally integrated with the exception of the lower two cassettes (P_{GAL1} -*BtispG* and P_{BIO2} -*ispD*) which were housed on a 2-micron plasmid. See the Supplementary Material for further details on selection markers and integration loci. The parent strain, Y5823, is *S. cerevisiae* CEN.PK2 *erg13::LEU2*, PRM10 (L156Q).

Fig. 4. Determination of carbon flux via the DXP pathway. (A) Schematic of ^{13}C -labeling strategy. Dashed lines indicate multiple steps. (B) Mass spectrum showing ^{13}C -labeling patterns in ergosterol from strain Y6084/pAM2398-*ispD*. The inset mass spectrum shows the pattern for ergosterol from Y6084, which lacks *ispG*. Both cultures were provided with $^{13}\text{C}_6$ -labeled galactose and unlabeled mevalonate, as described.

Fig. 5. Further improvement of flux via the DXP pathway. (A) Improvement of flux via the DXP pathway (as shown by percentage of ergosterol that is ^{13}C -labeled) by reduction of aeration and addition of an extra copy of *ispD*. Y6084 is the parent strain, containing all of the DXP pathway genes except *ispG*. Y6084-a is Y6084 containing pAM2398 (*BtispG*), and Y6084-b is Y6084 containing pAM2398-*ispD* (adding a second copy of *ispD*). Culture shaking speeds are indicated. (B) Abundance of DXP pathway intermediates (relative to strain Y6084) in response to increasing DXP pathway flux. Culture labeling is as per (A). CDP-MEP and HMBPP were not detected. Error bars represent the standard deviation from three biological replicates.

Fig. 6. Growth of *S. cerevisiae* supported by the DXP pathway. Strain Y6084-a is Y6084 containing pAM2398 (*BtispG*), and strain Y6084-b is Y6084 containing pAM2398-*ispD* (adding a second copy of *ispD*). Cultures were cultured in SD medium containing either 15 mM mevalonate (mev) or no mevalonate. Error bars represent the standard deviation from three biological replicates.

A.

B.

A.

B.

Table 1. Demonstration of flux to ergosterol via the DXP pathway. All strains were constructed by the addition of cassettes encoding the IspG, IspH, and Fld enzymes and fusion proteins listed below either by chromosomal integration (G) or on 2-micron plasmids (2 μ) into Y6081, which harbors the top portion of the DXP pathway.

Strain	IspG	IspH	% Ergosterol labeled
Y6544	2 μ : P _{GAL1} - <i>BtispG-Bsfld</i>	G: P _{GAL1} - <i>EcfldA-EcispH</i>	1.35 +/- 0.01
Y6540	G: P _{GAL1} - <i>BtispG-Bsfld</i>	G: P _{GAL1} - <i>EcfldA-EcispH</i>	1.62 +/- 0.03
Y6533	G: P _{GAL1} - <i>BtispG-Bsfld</i>	G: P _{GAL1} - <i>EcfldA-EcispH</i> (integrated at <i>GAL80</i>)	1.66 +/- 0.08
Y6084/ pAM2397	G: P _{GAL1} - <i>Bsfld</i> 2 μ : P _{GAL1} - <i>BsispG</i>	G: P _{GAL1} - <i>EcispH</i>	5.04 +/- 0.23
Y6084/ pAM2398	G: P _{GAL1} - <i>Bsfld</i> 2 μ : P _{GAL1} - <i>BtispG</i>	G: P _{GAL1} - <i>EcispH</i>	5.27 +/- 0.04