ARTICLE

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Redirection of Flux Through the FPP Branch-Point in *Saccharomyces cerevisiae* by Down-Regulating Squalene Synthase

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ABSTRACT: *Saccharomyces cerevisiae* utilizes several regulatory mechanisms to maintain tight control over the intracellular level of farnesyl diphosphate (FPP), the central precursor to nearly all yeast isoprenoid products. High-level production of non-native isoprenoid products requires that FPP flux be diverted from production of sterols to the heterologous metabolic reactions. To do so, expression of the gene encoding squalene synthase (*ERG9*), the first committed step in sterol biosynthesis, was down-regulated by replacing its native promoter with the methionine-repressible *MET3* promoter. The intracellular levels of FPP were then assayed by expressing the gene encoding amorphadiene synthase (*ADS*) and converting the FPP to amorphadiene. Under certain culture conditions amorphadiene production increased fivefold upon *ERG9* repression. With increasing flux to amorphadiene, squalene and ergosterol production each decreased. The levels of these three metabolites were dependent not only upon the level of *ERG9* repression, but also the timing of its repression relative to the induction of *ADS* and genes responsible for enhancing flux to FPP.

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Introduction

With over 50,000 identified members, terpenoids comprise the largest class of natural products (Conolly and Hill, 1991). Great structural diversity is found among these compounds, allowing them to perform a variety of essential biochemical functions processes such as electron transport, maintaining membrane fluidity, subcellular protein targeting and regulation, and cellular and organismal development. Moreover, the value of these small molecules extends beyond their biological utility—they have been commercialized to serve as antibiotics, pest control agents in agriculture, fragrances and flavors, and important anticancer and pharmaceutical products.

A specific class of these natural products, sesquiterpenoids, is derived from the same 15-carbon building block farnesyl diphosphate (FPP). These C_{15} molecules include such compounds as nootkatone (an important component in grapefruit flavor), bisabolol (an additive in many cosmetics), and nerolidol (a fragrance additive for soap). Perhaps the most important sesquiterpenoid is artemisinin, an effective anti-malarial drug that is synthesized in the plant *Artemisia annua* by oxidation of the sesquiterpene olefin amorphadiene. Unfortunately, the cost of cultivation and extraction from this plant supply makes artemisinin unaffordable to many malaria sufferers.

As a first step towards an alternative route for artemisinin synthesis, amorphadiene synthase (*ADS*) was introduced into *Saccharomyces cerevisiae*, enabling the conversion of a portion of the native FPP metabolic pool to amorphadiene (Ro et al., 2006). With the goal of increasing amorphadiene

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titers, the pool of FPP available to ADS needed to be maximized. FPP is the last common precursor for almost all terpenoid production (e.g., sterols, prenylated proteins, and dolichols) in yeast and is thus at the intersection of many branching metabolic pathways (Fig. 1). As such, FPP is an important feed-back regulator of its synthesis via the mevalonate pathway (the sole route of terpenoid production in all fungi) (Dimster-Denk et al., 1999). By previously modulating the expression of several enzymes important for determining the flux through the mevalonate pathway to FPP, we greatly enhanced the amorphadiene production capacity of a yeast strain (Ro et al., 2006). By limiting the flux of FPP to the competing sterol pathway, titers were further improved. Herein, we focus on the redirection of carbon flux away from sterols and towards amorphadiene production.

Squalene synthase (SQS), encoded by *ERG9*, catalyzes the first committed step of sterol synthesis and plays a key role in determining the flux through the FPP branch point. It has been suggested that of the various FPP products, only sterol production is affected by fluctuations in the intracellular FPP concentration. According to this theory, FPP flow is diverted to squalene synthase because of a predicted lower affinity for FPP (Goldstein and Brown, 1990). The other enzymes at the FPP branch point, *cis-* and *trans-*prenyltransferases, have higher affinities and would thus be fully saturated even at low FPP concentrations. Grabowska et al. (1998), however, have disputed this hypothesis by showing that disruptions in *ERG9,* which increase the available FPP pool, led to a sixfold increase in polyprenol synthesis. Accordingly, they instead proposed that it is SQS that is responsible for directing FPP flux to sterol production, possibly by metabolite channeling between SQS and FPP synthase combined with a short half-life of SQS.

By using the methionine-repressible *MET3* promoter to drive *ERG9*, we were able to achieve tunable control over flux to the sterol pathway. Incorporation of this construct into strains engineered with different FPP-production capacities allowed us to evaluate the impact of limited flux to sterol synthesis on amorphadiene production in each FPP context. We also found that not only is the level of *ERG9* repression important to maximizing amorphadiene production, but so is the timing of this repression relative to the induction of genes responsible for enhancing flux through the mevalonate pathway to FPP. These studies broaden our understanding of how FPP is rerouted from sterol synthesis to amorphadiene synthesis when *ERG9* is repressed, which may lead to further strain improvements in the future.

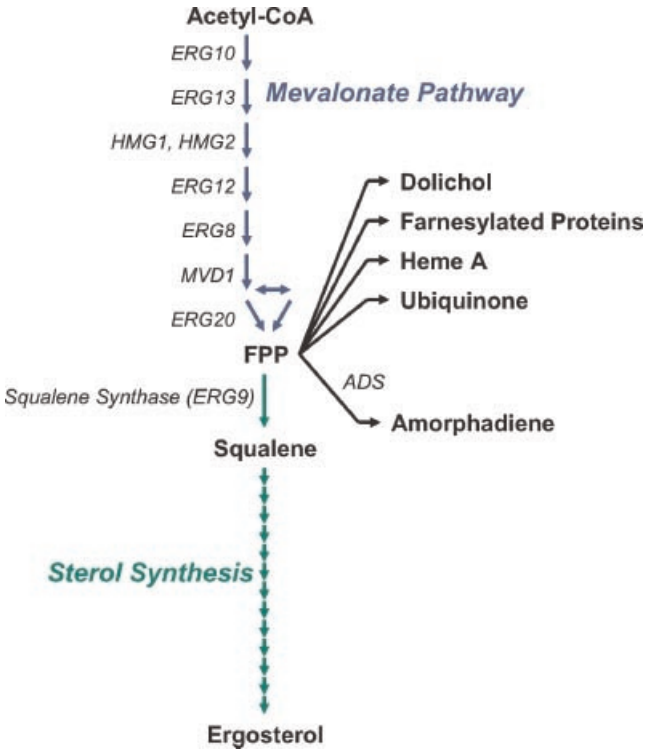

Figure 1. The FPP branch-point in *S. cerevisiae*. FPP is derived from the mevalonate pathway and utilized in the synthesis of dolichol, prenylated proteins, heme A, ubiquinone, and sterols (via the synthesis of squalene). *ADS* has been introduced to catalyze the formation of amorphadiene from FPP. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Materials and Methods

Chemicals

Dodecane and caryophyllene were purchased from Sigma–Aldrich (St. Louis, MO). Complete Supplement Mixtures for formulation of Synthetic Defined (SD) media were purchased from Qbiogene (Irvine, CA). All other media components were purchased from either Sigma–Aldrich or Becton, Dickinson (Franklin Lakes, NJ).

Strains and Media

S. cerevisiae BY4742 (Carrie Baker Brachmann et al., 1998), a derivative of S288C, served as the parent strain for all yeast strains constructed here. This strain was grown in rich YPD medium (Burke et al., 2000). Engineered yeast strains were grown in SD medium (Burke et al., 2000) with leucine, uracil, histidine, and/or methionine dropped out where appropriate. For induction of genes expressed from the *GAL1* promoter, *S. cerevisiae* strains were grown with galactose and glucose at the amounts indicated.

Yeast Transformation and Strain Construction

Transformation of all strains of *S. cerevisiae* was performed by the standard lithium acetate method (Gietz and Woods,

2002). Three to ten colonies from each transformation were screened for the selection of the highest amorphadiene-producing transformant. Strains EPY201, EPY225, EPY213, and EPY224 (Table I) used in this study were constructed in an earlier study and have been described previously (Ro et al., 2006). To replace the *ERG9* promoter with the *MET3* promoter, plasmid pRS-ERG9 (Ro et al., 2006)—which contains a truncated 5′ segment of *ERG9* placed in front of *MET3* promoter (Gardner and Hampton, 1999)—was used. Plasmid pRS-ERG9, which contains the selectable *HIS3* marker, was cleaved with *Hinc*II for the integration of the P_{MET3}-*ERG9* fusion at the *ERG9* locus of EPY201 for the construction of EPY226 (Table I).

Yeast Cultivation

All optical density at 600 nm (OD_{600}) measurements were taken using a Beckman DU-640 spectrophotometer. For time course experiments to measure amorphadiene production, culture tubes containing 5 mL of SD (supplemented with 2% galactose or 2% glucose, as indicated) medium with LEU and MET omitted (HIS was also omitted for strains EPY226, EPY225, and EPY213) were used as starter cultures. These cultures were grown at 30°C to OD_{600} between 1 and 2 and then used to inoculate 250-mL unbaffled culture flasks containing 50 mL SD medium (2% galactose or 0.2% glucose/1.8% galactose, as indicated; LEU and HIS omitted as appropriate; MET added as indicated) at an OD_{600} of 0.05. Because amorphadiene at very low concentrations is volatile from aqueous cultures (Newman et al., 2005), 5 mL dodecane was added to each culture flask in order to sequester and retain the amorphadiene as it was produced. Ten microliters of the immiscible dodecane layer was sampled and diluted 100-fold in ethyl acetate for determination of amorphadiene production by GC–MS.

GC–MS Analysis of Metabolites

GC–MS analysis was used to measure amorphadiene production from yeast cultures. Samples were run using a previously described method (Martin et al., 2001) with some differences: The GC oven temperature program used a 100°C for 0.75 min, followed by a ramp to 300°C at 50°C/min, and a 1 min hold at 300°C. Injector and MS quadrupole detector temperatures were 230°C and 150°C, respectively. The MS was operated in selected ion monitoring (SIM) mode using ions of *m/z* 204 and 189, which represent the molecular ion and an abundant fragment ion ($[M-CH_3]^+$) of amorphadiene, respectively. Amorphadiene concentrations were calculated in (−)-*trans*-caryophyllene equivalents using a caryophyllene standard curve and the relative abundance of ions 189 and 204 *m/z* to their total ions. The sesquiterpene caryophyllene was purchased from Sigma–Aldrich.

Squalene and ergosterol were also extracted from yeast cultures, and concentrations were measured using GC–MS analysis. The cells from 1 mL of yeast culture were pelleted and washed once with water. The pellet was lysed in an alcoholic potassium hydroxide solution (20% (w/v) KOH in 50% EtOH) by boiling for 5 min. After cooling, the sterols were extracted with dodecane. Cholesterol was present in the alcoholic KOH solution and was used as an internal standard for GC–MS analysis. The same instrumentation was used in this analysis as described for the amorphadiene quantification. Samples from a splitless, 1-μL injections were separated using a GC oven temperature program of 80°C for 1 min, then a ramp to 280°C at 20°C/min with a 20 min hold time at 280°C, followed by a 20°C/min ramp to 300°C, with a final hold time for 2 min. Ion source, MS quadrupole detector, and inlet temperatures were 300, 200, and 250°C, respectively. The MS was operated in selected ion monitoring (SIM) mode using ions of *m/z* 149, 203, 218, 363, which are representative ions of squalene, ergosterol, and cholesterol. Squalene and ergosterol concentrations were calculated using standards purchased from Sigma–Aldrich.

Results

Squalene Synthase Repression

To decrease the amount of FPP routed to sterol synthesis, flux to squalene was limited by down-regulating expression of *ERG9*, encoding squalene synthase. As an *ERG9* deletion is lethal without exogenous supplementation of sterols, *ERG9* was transcriptionally down-regulated by replacing its native

Table I. Yeast strains used in this study.

Name	Genotype	Description	References
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*		Brachmann et al. (1998)
EPY201	BY4742 pRS425ADS	*ADS* high-copy expression	Ro et al. (2006)
EPY226	EPY201 *erg9::*P_{MET3}-*ERG9* (ura-)	As per EPY 201 plus *ERG9* promoter replacement with P_{MET3}	Gardner and Hampton (1999), Gardner et al. (2001)
EPY225	EPY226 P_{GAL1}-*tHMGR* (ura-)	As per EPY226 plus *tHMG1* overexpressed from the genome	Donald et al. (1997), Ro et al. (2006)
EPY213	EPY225 P_{GAL1}-*upc2-1* (ura-)	As per EPY225 plus *upc2-1* overexpressed from the genome	Davies et al. (2005), Ro et al. (2006)
EPY224	EPY213 P_{GAL1}-*tHMGR* P_{GAL1}-*ERG20* (ura-)	As per EPY213 plus an additional copy of *tHMG1* and *ERG20* overexpressed from the genome	Szkopinska et al. (2000), Ro et al. (2006)

promoter with a methionine-repressible promoter, P_{MET3} (Cherest et al., 1985), a strategy that Gardner et. al. previously utilized to study HMGR degradation signals (Gardner and Hampton, 1999; Gardner et al., 2001). pRS-ERG9 was integrated into several engineered yeast strains, that vary in their capacity for FPP production (Table I). EPY201, EPY225, EPY213, and EPY224 in this table had been constructed in a previous study (Ro et al., 2006) for the purpose of increasing metabolic flux through the mevalonate pathway (Fig. 1). These strains, in addition to EPY226, vary in their capacity for FPP production and all contain a mechanism (P_{MET3}-*ERG9* integration) to modulate *ERG9* expression.

EPY201 contains only a P_{GAL1}-*ADS* expression cassette on a high-copy plasmid and produces only a limited amount of amorphadiene after 5 days of cultivation (Fig. 2). EPY226 has the same inherent limitations to FPP production as EPY201, but contains the P_{MET3}-*ERG9* fusion at the ERG9 locus.

EPY225 contains a P_{GAL1}-*tHMGR* expression cassette integrated into the chromosomal DNA of EPY226. *tHMGR* is a truncated HMG-CoA reductase (HMGR) sequence coding for only the catalytic domain of this enzyme (Donald et al., 1997). Overexpression of *tHMGR* using the *GAL1* promoter circumvents the transcriptional repression by FPP for this key regulatory checkpoint of the mevalonate pathway.

EPY213 contains a P_{GAL}-*upc2-1* expression cassette integrated into the chromosomal DNA in addition to *tHMGR* to further increase flux through the mevalonate pathway. *upc2-1* is a semi-dominant mutant allele that enhances the activity of UPC2 (a global transcription factor regulating the biosynthesis of sterols in *S. cerevisiae*) (Davies et al., 2005). Binding motifs for this transcription factor are found upstream of several genes of the mevalonate pathway (*ERG8*,*12*,*13*) as well as genes involved in sterol biosynthesis from FPP (*ERG1*,*6*,*11*,*25*,*2*,*3*) (Vik and Rine, 2001). In addition, UPC2 activates the expression of many other genes, most notably some *DAN/TIR* genes, which are hypoxically induced genes responsible for changes in the yeast cell wall (Abramova et al., 2001a,b; Davies and Rine, 2006).

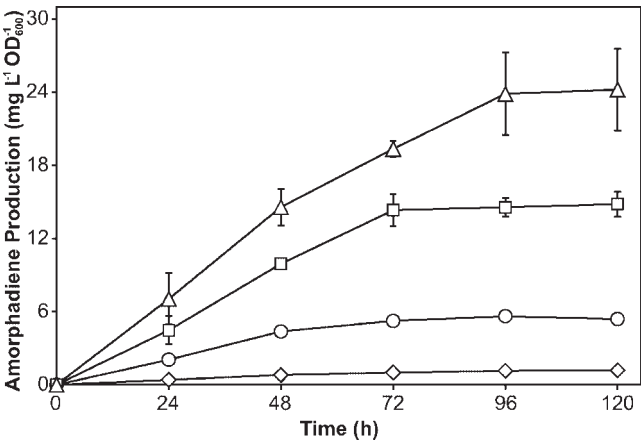

Figure 2. Amorphadiene production for strains with an integrated P_{MET3}-*ERG9* cassette. Production of amorphadiene is shown over 120 h by *S. cerevisiae* EPY201 (◇), EPY226 (○), EPY225 (□), and EPY213 (△). All cultures contained 1 mM methionine. The data, shown as specific production (production normalized for OD_{600}), are means ± standard deviations for three independent cultures.

EPY201, EPY226, EPY225, and EPY213 were cultured for 120 h in an SD-LEU medium containing a mixed carbon source (0.2% glucose/1.8% galactose) and supplemented to 1 mM methionine. It has been reported that the *MET3* promoter in yeast strain YPH499 exhibits a dynamic range of activity at low methionine concentrations with minimal expression above 0.1 mM (Mao et al., 2002). A 1 mM level of methionine was therefore selected to ensure minimal expression from the *MET3* promoter in our strains constructed in the BY4742 genetic background. Amorphadiene concentrations were measured every 24 h and normalized for cell density (Fig. 2). EPY201, which contains a plasmid bearing *ADS* but no modifications to the mevalonate pathway, produced 1 $mg\,L^{-1}\,OD_{600}^{-1}$ amorphadiene. Introduction of the P_{MET3}-*ERG9* fusion into this background (EPY226) increased production to 5 $mg\,L^{-1}\,OD_{600}^{-1}$. The subsequent addition of an expression cassette for *tHMGR* (EPY225) further increased production to 15 $mg\,L^{-1}\,OD_{600}^{-1}$. Finally, the addition of an expression cassette for *upc2-1* (EPY213) resulted in a production level of 24 $mg\,L^{-1}\,OD_{600}^{-1}$.

Relative Contribution of *ERG9* Repression to Amorphadiene Production

The *MET3* promoter should provide maximum transcription without methionine present in the medium. We cultured strains EPY201, EPY226, EPY225, and EPY213 in the absence of methionine and compared the amorphadiene levels at 120 h to cultures grown in 1 mM methionine (Fig. 3A). This comparison allowed us to discern the contribution that *ERG9* repression has on amorphadiene production relative to the other modifications made to the strains.

As anticipated, the methionine alone had no impact on EPY201, which contains an unaltered *ERG9* gene driven by the native promoter. Addition of 1 mM methionine to cultures of EPY226, which harbors the methionine-repressible P_{MET3}-*ERG9* fusion, resulted in a 2.3-fold increase in amorphadiene production. A more dramatic 4.1-fold change was observed in the higher FPP-producing EPY225 strain. These data suggest that the full impact of *tHMGR* expression is only realized when *ERG9* activity is also limited—with full activity of squalene synthase, the additional flux to FPP will be consumed by the sterol pathway or stored as squalene in lipid particles (Polakowski et al., 1999). Although the additional expression of *upc2-1* in EPY213 did enhance the strain's capacity to produce

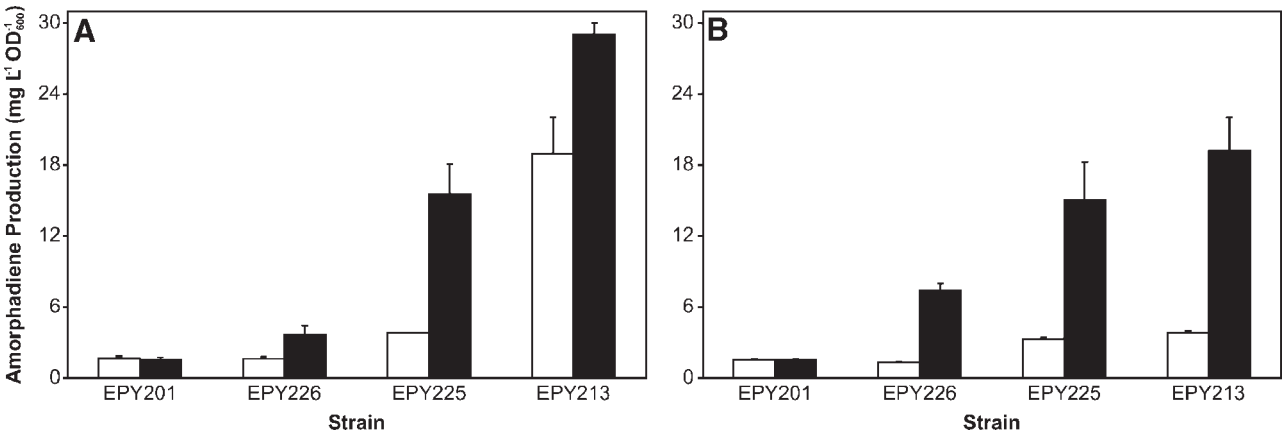

Figure 3. Effect of methionine addition on amorphadiene production from strains containing a P_{MET3}-*ERG9* cassette. Production of amorphadiene at 120 h by *S. cerevisiae* EPY201, EPY226, EPY225, and EPY213. Each strain was grown at 0 mM methionine (white bars) as well as at 1 mM methionine (filled bars). **A**: Strains were grown in a mixed carbon medium containing 0.2% glucose and 1.8% galactose. **B**: Strains were also grown in a pure galactose medium containing 2% galactose. The data, shown as specific production (production normalized for OD_{600}), are means ± standard deviations for three independent cultures.

amorphadiene, the repression of *ERG9* surprisingly led to only a 50% increase in amorphadiene production.

Timing of *ERG9* Repression

The work described to this point has used a medium with a mixed carbon source. A seed culture for each strain that was initially grown in a medium with 2% glucose was used to inoculate a production culture containing 0.2% glucose and 1.8% galactose. Inoculating a pure galactose medium from a 2% glucose seed culture resulted in a lengthy lag phase as the cells adjust to the new carbon source. It was observed that the lag phase was exaggerated further in our engineered strains containing additional genes, particularly *upc2-1*, under the transcriptional control of *GAL* promoters. The concentrations of glucose and galactose (0.2% and 1.8%, respectively) were selected because they alleviated this lag period while maximizing amorphadiene production (data not shown). Because methionine was added at inoculation, while glucose was present in the medium, the *ERG9* was subject to repression prior to the depletion of glucose and induction of ADS, *tHMGR*, and/or *upc2-1*. To examine the effect of delaying *ERG9* repression until after the full induction of *ADS*, *tHMGR*, and/or *upc2-1*, the engineered yeasts strains were initially cultured in a medium containing 2% galactose. These seed cultures were then used to inoculate larger production cultures containing 2% galactose and either 0 or 1 mM methionine. Again, amorphadiene concentrations were measured at 120 h and compared for each of these conditions (Fig. 3B).

As seen with the mixed carbon source, methionine had no impact on EPY201. The repression of *ERG9* expression by methionine in EPY226 resulted in a 5.6-fold increase in amorphadiene production, over twice the difference observed with the mixed carbon cultures. The addition of methionine increased amorphadiene production in EPY225 and EPY213 by 4.6- and 5-fold, respectively. The change observed for EPY213 was significantly greater than that the 50% increase seen when a mixed carbon source was used. So, delaying the repression of *ERG9* until after *tHMGR* and *upc2-1* were fully induced (and the cells had time to adapt to the changes invoked by induction of the *upc2-1* transcription factor) resulted in a larger pool of FPP available for redirection from sterol synthesis amorphadiene production.

Tunable Down-Regulation of *ERG9* for Maximizing Amorphadiene Production

The MET3 promoter exhibits a tunable expression level dependent upon the level of methionine supplied to the culture (Mao et al., 2002). To ensure the full range of transcriptional activity from the P_{MET3}-*ERG9* fusion was covered, we supplemented cultures of strain EPY213 with total methionine concentrations ranging from 0 to 2 mM. These methionine concentrations were used in mixed carbon cultures as well as pure galactose cultures as described in the previous section, and amorphadiene production was measured every 24 h for 120 h (Fig. 4).

In the pure galactose cultures, maximum amorphadiene production was reached at methionine concentrations above 0.2 mM, representing in a 3.3-fold improvement in total amorphadiene production over cultures with no methionine. More interestingly, the mixed carbon cultures exhibited maximum amorphadiene production at 120 h when supplied with 0.2 mM methionine (1.7-fold higher than when no methionine was added) with production levels dropping at higher methionine concentrations. The data from the pure galactose cultures support previous data showing minimal expression from the *MET3* promoter at a methionine concentration of 0.2 mM (Mao et al., 2002),

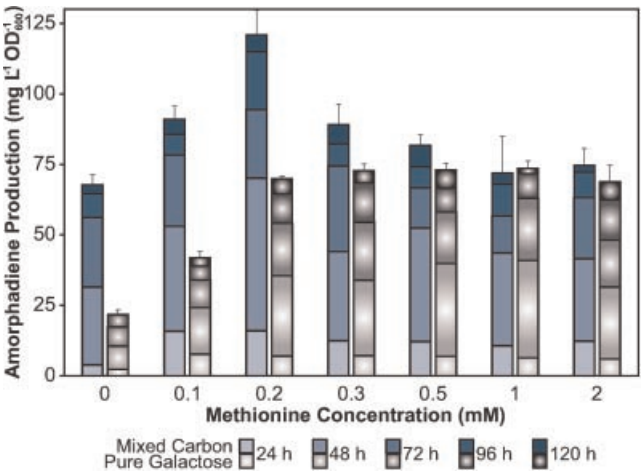

Figure 4. Amorphadiene production in strain EPY213 at various levels of methionine. The strain was grown in a mixed carbon medium containing 0.2% glucose and 1.8% galactose (blue) as well as in a pure galactose medium containing 2% galactose (red). Production of amorphadiene is shown over 120 h for strains cultured at seven different methionine concentrations. The data, shown as total production, are means ± standard deviations for three independent cultures. The error bars represent the standard deviation at the 120 h time point. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

suggesting that a factor other than the extent of *ERG9* repression contributes to attaining maximum amorphadiene production using the mixed carbon medium. Although these data are reported in total production per volume, the specific production normalized for cell density follows a similar trend. However, marginally (as much as 15%) lower cell densities were observed for methionine concentrations of 0.3 mM or greater.

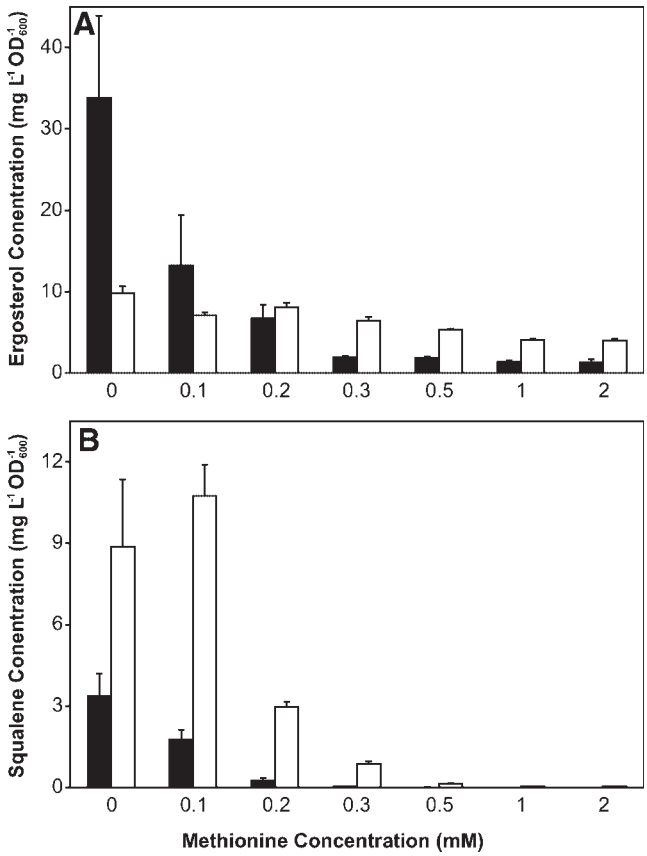

Figure 5. Ergosterol and squalene production in strain EPY213 at various levels of methionine. EPY213 was cultured at seven different methionine concentrations. Ergosterol (**A**) and squalene (**B**) concentrations were measured at 120 h a mixed carbon medium containing 0.2% glucose and 1.8% galactose (filled bars) as well as in a pure galactose medium containing 2% galactose (white bars). These data are shown as means ± standard deviations for three independent cultures.

Ergosterol and Squalene Production

We hypothesized that the slightly lower cell densities observed at higher methionine concentrations may be due to insufficient flux to ergosterol or other sterols. Ergosterol, which is important for maintaining proper membrane fluidity in *S. cerevisiae*, as well as squalene, the first committed metabolite in sterol synthesis, were measured at 120 h in the cultures described in the previous section (Fig. 5). For the mixed carbon cultures, a dramatic decrease in both ergosterol and squalene concentrations was observed between 0 and 0.3 mM methionine. A similar decrease was also seen for squalene using the pure galactose medium; however, the methionine concentration had a less marked impact on the measured ergosterol content of the cells. It is also interesting to note that at methionine concentrations below 0.3 mM the pure galactose cultures produced comparable or lower quantities of ergosterol but significantly more squalene than the mixed carbon cultures. Ergosterol and squalene were also measured every 24 h up to 120 h with a similar trend observed for each metabolite as seen at 120 h (data not shown). The inverse relationship observed here between the squalene/ergosterol and amorphadiene concentrations supports the down-regulation of *ERG9* with the addition of methionine.

Discussion

Previously, we engineered strains for enhanced flux to and through the mevalonate pathway for increased terpenoid production (Ro et al., 2006; Shiba et al., 2006). Here, we specifically examined limiting the activity of the first committed step in sterol synthesis and its impact on the cell and amorphadiene production. For this purpose, we engineered yeast strains with different capacities for FPP production to explore the redirection of flux away from the sterol pathway and towards amorphadiene for each strain context.

Using the methionine-repressible *MET3* promoter in the P_{MET3}-*ERG9* fusion (Gardner and Hampton, 1999; Gardner et al., 2001) is an ideal method for lowering the

activity of squalene synthase. Mao et al. (2002) demonstrated that methionine concentrations of 0–0.2 mM modulated expression of P_{MET3}-*lacZ* over a 16-fold range; yet even at the maximum level of repression, the P_{MET3}-*ERG9* fusion maintained sufficient squalene synthase activity for normal cell growth.

Although the expression of *tHMGR* and *upc2-1* increased flux to FPP as indicated by our amorphadiene assay, the full effect of these modifications may not be realized in a strain with several other enzymes competing for the common FPP pool (Fig. 1). By limiting the squalene synthase activity, we observed significant yield increases in amorphadiene, particularly for the pure galactose cultures. In addition to yield increases from redirection of FPP flux away from sterol synthesis, regulatory effects related to the depletion of sterols could also contribute to the greater amorphadiene yields observed. Ergosterol and sterol intermediates have been observed to play a role in feedback regulation of some mevalonate pathway enzymes (Dimster-Denk and Rine, 1996; Gardner et al., 2001; Goldstein and Brown, 1990; Servouse and Karst, 1986), and artificial depletion of these sterols could increase flux through the mevalonate pathway to FPP by reducing feedback inhibition.

We also found that the relative timing between the repression of *ERG9* and the induction of *ADS*, *tHMGR*, and/or *upc2-1* played a significant role in determining total amorphadiene accumulation. As mentioned, the relative increase in amorphadiene production resulting from the repression of *ERG9* is more pronounced when the engineered strains were cultivated with galactose as the sole carbon source. In these cultures, the cells had already adapted to galactose, and *ADS*, *tHMGR*, and/or *upc2-1* were fully induced before the down-regulation of *ERG9*. Thus, the constriction of flux to sterols elicited by the addition of methionine was temporally coordinated with the shift in cell metabolism in order to maximize the diversion of FPP to amorphadiene.

In mixed carbon cultures, expression from the *GAL* promoters that drive *ADS*, *tHMGR*, and/or *upc2-1* expression was repressed until glucose was consumed. During this initial growth phase on glucose, the early *ERG9* repression by methionine, could have caused a temporary accumulation of FPP since ADS was not present to catalyze its conversion to amorphadiene. As a major feedback regulator of the mevalonate pathway (Dimster-Denk et al., 1999), accumulation of FPP may inadvertently limit flux through the pathway through a regulatory mechanism, ultimately leading to lower amorphadiene production.

The greatest difference in *ERG9* repression between cultivation methods was seen with EPY213, which expresses *upc2-1* upon galactose induction. The fact that the increase in amorphadiene levels upon *ERG9* repression is not as pronounced in the mixed carbon medium could be largely due to a inherent higher capacity for FPP production by this strain under these growth conditions. The mixed carbon medium minimized the lag period associated with metabolism changes and *ADS/tHMGR/upc2-1* induction, eliminating the need for pre-culture growth in galactose. Expression of *ADS*, *tHMGR*, and *upc2-1* were not induced in the glucose pre-culture and were not even fully induced until the glucose in the production culture was consumed (occurring at approximately 12 h post-inoculation, data not shown). Limiting the window of *upc2-1* expression is likely to be most beneficial for the cells, for the transcription factor confers further physiological changes by modulating gene expression beyond just the mevalonate pathway (Davies and Rine, 2006). Limiting the window of *upc2-1* expression ensures that any negative affects associated with its overexpression were curtailed, and thus we observed higher titers of amorphadiene at both 0 and 1 mM levels of methionine compared to the pure galactose cultures. Plasmid stability is another factor that likely contributes to the production difference between the two carbon source regimes. We have found that the high-copy pRS425ADS plasmid is retained by cells cultured in the mixed carbon medium to more than twice the extent of cells cultured in the pure galactose medium (supplementary data). The earlier induction of the *GAL1* promoter in the pure galactose precultures is the most likely cause of the higher plasmid instability, for most of the difference in plasmid stability between the carbon source regimes was observed even prior to inoculation of the production cultures (supplementary data).

With many factors contributing to the level of amorphadiene production in EPY213, we examined a range of methionine concentrations designed to modulate the level of *ERG9* repression. The cultures grown in galactose showed a predictable trend in which a methionine concentration of 0.3 mM (sufficient for maximal *ERG9* repression) maximized amorphadiene production, and higher methionine concentrations maintained this maximum level of production. The mixed carbon cultures, however, showed a more interesting pattern where a 0.2 mM methionine concentration maximized amorphadiene production and higher concentrations resulted in lower amorphadiene production. As discussed previously, timing of the *ERG9* repression relative to induction of the other genes in EPY213 appears to be an important factor in determining the total flux through the mevalonate pathway to FPP and amorphadiene. Higher methionine concentrations in the medium may result in a more rapid accumulation within the cell and, subsequently, earlier repression of *MET3* transcription. The intermediate (0.2 mM) methionine concentration appears to be sufficient to limit *ERG9* expression, but the effect may be slow enough to mitigate some of the negative impacts of repressing *ERG9* prior to induction of *ADS*, *tHMGR*, and/or *upc2-1*.

It was surprising that the reduced squalene synthase activity did not have a larger impact on cell growth; the reduction in final cell density observed when cultures were supplemented with 1 or 2 mM methionine was no greater than 15%. The metabolic product of ERG9, squalene, as well as the final product of the sterol pathway, ergosterol, were each measured in EPY213 across the given range of methionine concentrations. For cells grown on either

carbon source, squalene concentration generally followed the expected inverse relationship to amorphadiene levels, where squalene decreased with increasing repression of *ERG9*. The ergosterol levels followed this same trend as flux to sterol synthesis was increasingly constricted. The pure galactose cultures, however, displayed only a marginal decrease in ergosterol accumulation compared to the mixed carbon cultures. This difference may be attributed to the fact that such parameters as media composition and growth alone can significantly affect ergosterol accumulation in yeast cells (Arnezeder and Hampel, 1990).

These studies have shown that, in addition to factors affecting promoter strength, the relative timing of the induction or repression of independent promoter systems can be crucial when attempting to balance the numerous components of a metabolic pathway.

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