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Optimization of a heterologous mevalonate pathway through the use of variant HMG-CoA reductases

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ABSTRACT

Expression of foreign pathways often results in suboptimal performance due to unintended factors such as introduction of toxic metabolites, cofactor imbalances or poor expression of pathway components. In this study we report a 120% improvement in the production of the isoprenoid-derived sesquiterpene, amorphadiene, produced by an engineered strain of *Escherichia coli* developed to express the native seven-gene mevalonate pathway from *Saccharomyces cerevisiae* (Martin et al. 2003). This substantial improvement was made by varying only a single component of the pathway (HMG-CoA reductase) and subsequent host optimization to improve cofactor availability. We characterized and tested five variant HMG-CoA reductases obtained from publicly available genome databases with differing kinetic properties and cofactor requirements. The results of our *in vitro* and *in vivo* analyses of these enzymes implicate substrate inhibition of mevalonate kinase as an important factor in optimization of the engineered mevalonate pathway. Consequently, the NADH-dependent HMG-CoA reductase from *Delftia acidovorans*, which appeared to have the optimal kinetic parameters to balance HMG-CoA levels below the cellular toxicity threshold of *E. coli* and those of mevalonate below inhibitory concentrations for mevalonate kinase, was identified as the best producer for amorphadiene (54% improvement over the native pathway enzyme, resulting in 2.5 mM or 520 mg/L of amorphadiene after 48 h). We further enhanced performance of the strain bearing the *D. acidovorans* HMG-CoA reductase by increasing the intracellular levels of its preferred cofactor (NADH) using a NAD^+-dependent formate dehydrogenase from *Candida boidinii*, along with formate supplementation. This resulted in an overall improvement of the system by 120% resulting in 3.5 mM or 700 mg/L amorphadiene after 48 h of fermentation. This comprehensive study incorporated analysis of several key parameters for metabolic design such as *in vitro* and *in vivo* kinetic performance of variant enzymes, intracellular levels of protein expression, in-pathway substrate inhibition and cofactor management to enable the observed improvements. These metrics may be applied to a broad range of heterologous pathways for improving the production of biologically derived compounds.

1. Introduction

Microbial production of desirable compounds through heterologous expression of foreign pathways commonly results in unintended consequences. Introduction of previously unknown and potentially toxic metabolites in the host organism may result in lower cell density and reduced product formation. Maintenance of a delicate balance between utilization of existing cellular resources for organism growth versus engineered manipulations such as enzyme overexpression is therefore an important aspect of metabolic optimization. Consequently, metabolic engineering strategies reported thus far have investigated accumulation of toxic intermediates (Berry et al., 2002; Pitera et al., 2007; Zhu et al., 2002), targeted improvements in protein production (Glick, 1995; Redding-Johanson et al., 2011), kinetics of rate limiting processes (Pfleger et al., 2006), spatial localization of key enzymatic activities (Chhabra and Keasling, 2011; Dueber et al., 2009; Pfleger et al., 2006; Zhang et al., 2008),

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and redox cofactor utilization by pathway components (Bennett and San, 2009; Berrios-Rivera et al., 2002; San et al., 2002).

The isoprenoid biosynthetic pathway is an important source of biopharmaceuticals, biochemicals, and advanced biofuels (Fortman et al., 2008). In nature, terpenoids are synthesized from the universal precursors isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), which are generated either through the mevalonate (MEV) pathway or the deoxyxylulose 5-phosphate (DXP) pathway (Bochar et al., 1999; Hedl et al., 2004; Kuzuyama, 2002; Wilding et al., 2000). Generally, Gram-negative bacteria and eukaryotic organelles employ the DXP pathway, while humans, mammals, other eukaryotes, archaea and gram-positive cocci utilize the enzymes and intermediates of the mevalonate pathway (Bochar et al., 1999; Hedl et al., 2004). In *Escherichia coli* and other gram-negative bacteria, IPP and DMAPP generated from the natively regulated DXP pathway are essential metabolites for the prenylation of tRNAs and the synthesis of farnesyl pyrophosphate, which is central for quinone and cell wall biosynthesis (Connolly and Winkler, 1989). Enhanced sesquiterpene production in *E. coli* has been accomplished through the heterologous expression of the MEV pathway from *Saccharomyces cerevisiae* thereby bypassing the regulatory effects of its native DXP pathway (Harada and Misawa, 2009; Martin et al., 2003; Pitera et al., 2007). While this foreign MEV pathway is potentially unregulated in *E. coli*, its presence affects cellular behavior in significant ways. Notably, accumulation of the pathway intermediate 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) has been shown to lead to bacteriostatic and bactericidal effects (Pitera et al., 2007). In addition, enzymatic conversion of HMG-CoA into mevalonate is the only redox cofactor utilization step in the entire MEV pathway and has the potential to disturb cellular redox balance (Berrios-Rivera et al., 2002; Heuser et al., 2007). The catalytic reaction performed at the HMG-CoA reductase step therefore represents a crucial bottleneck where metabolic engineering strategies may be applied to optimize the system for isoprenoid biosynthesis.

HMG-CoA reductase (HMGR), one of the few known four-electron oxidoreductases in nature, catalyzes the reduction of (*S*)-HMG-CoA to (*R*)-mevalonate (Fig. 1). Two moles of reduced pyridine nucleotide coenzyme are oxidized during the reduction of 2 mol of the thioester group of HMG-CoA to the primary hydroxyl group of mevalonate. HMGR is also able to catalyze the reverse reaction, the oxidative acylation of (*R*)-mevalonate to (*S*)-HMG-CoA. Based on sequence divergence, two classes of HMGRs have been proposed (Bischoff and Rodwell, 1992; Boucher et al., 2001; Istvan, 2001; Rodwell et al., 2000). Conventionally, class I HMGRs are typically found in higher organisms, generally prefer NADPH as the cofactor (Lawrence et al., 1995), and under physiological conditions, are effectively irreversible even though the enzymes catalyze the reaction in both directions *in vitro* (Bach et al., 1986; Lawrence et al., 1995; Sherban et al., 1985). In contrast, class II HMGRs comprise those from prokaryotes and archaea and generally prefer NADH as a cofactor (Kim et al., 2000; Theivagt et al., 2006).

It is well known that pathway optimization involves a complex interplay of factors such as: toxicity associated with newly introduced metabolites, disturbance in cellular redox state, *in vivo* enzymatic activity, and suboptimal protein expression levels. Previous efforts to alleviate the bottleneck at HMG-CoA reductase catalysis in microbial isoprenoid biosynthesis focused on manipulating features of only the native HMGR of the *S. cerevisiae* MEV pathway. These included modulation of enzyme production (Pitera et al., 2007), regulated enzyme production (Pfleger et al., 2006), and post-translational localization (Dueber et al., 2009; Zhang et al., 2008). The goal of this study was to alleviate the bottleneck associated with the HMG-CoA reduction step by perturbing enzymatic properties of HMGR while maintaining existing features of the engineered MEV pathway intact.

Our goal was to characterize enzymatic function in the context of a heterologous pathway expressed in an engineered host platform. We reasoned that direct assessment of such biochemical functionality would offer insight into the potential limitations of the engineered pathway and a rationale to resolve the corresponding bottlenecks. Here we exploited HMGR sequences from publicly available genome databases as a means to vary biochemical functionality of the reduction step while keeping the rest of the MEV pathway unchanged (Fig. 1). We investigated the kinetic characteristics of five HMGR variants individually as standalone enzymes and in the context of the entire MEV pathway to determine the ideal candidate for amorphadiene production. For the best performing HMGR variant, we further optimized amorphadiene production by improving preferred cofactor availability through the overexpression of a heterologous formate dehydrogenase. Our results emphasize the importance of

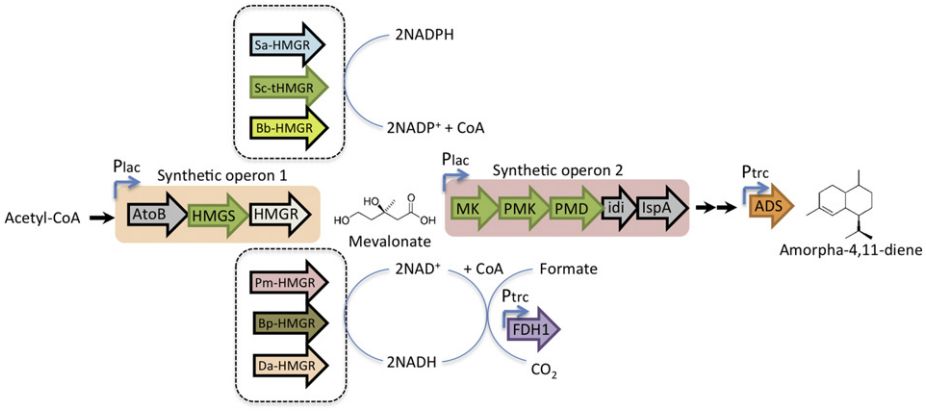

Fig. 1. Biosynthetic mevalonate pathway for isoprenoid production in *E. coli* towards amorpha-4,11-diene synthesis. Acetyl-CoA is diverted from central metabolism through the enzymes encoded in synthetic operon 1 to biosynthesize mevalonate. Synthetic operon 2 appropriates mevalonate to farnesyl pyrophosphate, which is subsequently extended to make the product, amorpha-4,11-diene. To increase intracellular pools of NADH the NAD^+-dependent formate dehydrogenase from *Candidia boidinii*, FDH yields a mole of NADH per mole of formate conversion to carbon dioxide. Genes originating from *E. coli* are colored in gray, genes originating from *S. cerevisiae* are in green, and the amorpha-4,11-diene synthase gene from *A. annua* is in orange. Organism abbreviations are as follows: *S. cerevisiae* (Sc), *P. mevalonii* (Pm), *S. aureus* (Sa), *B. petrii* (Bp), and *D. acidovorans* (Da). Gene abbreviations are as follows: acetoacetyl-CoA thiolase (*atoB*), HMG-CoA synthase (*HMGS*), HMG-CoA reductase (*HMGR*), mevalonate kinase (*erg12*, *MK*), phosphomevalonate kinase (*erg8*, *PMK*), phosphomevalonate decarboxylase (*MVD1*, *PMD*), IPP isomerase (*idi*), farnesyl diphosphate synthase (*ispA*), amorphadiene synthase (*ADS*), and formate dehydrogenase (*fdh1*).