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Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*

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Abstract

Engineering biosynthetic pathways in microbes for the production of complex chemicals and pharmaceuticals is an attractive alternative to chemical synthesis. However, in transferring large pathways to alternate hosts and manipulating expression levels, the native regulation of carbon flux through the pathway may be lost leading to imbalances in the pathways. Previously, *Escherichia coli* was engineered to produce large quantities of isoprenoids by creating a mevalonate-based isopentenyl pyrophosphate biosynthetic pathway [Martin, V.J., Pitera, D.J., Withers, S.T., Newman, J.D., Keasling, J.D., 2003. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat. Biotechnol. 21, 796–802]. The strain produces high levels of isoprenoids, but upon further investigation we discovered that the accumulation of pathway intermediates limited flux and that high-level expression of the mevalonate pathway enzymes inhibited cell growth. Gene titration studies and metabolite profiling using liquid chromatography–mass spectrometry linked the growth inhibition phenotype with the accumulation of the pathway intermediate 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA). Such an accumulation implies that the activity of HMG-CoA reductase was insufficient to balance flux in the engineered pathway. By modulating HMG-CoA reductase production, we eliminated the pathway bottleneck and increased mevalonate production. These results demonstrate that balancing carbon flux through the heterologous pathway is a key determinant in optimizing isoprenoid biosynthesis in microbial hosts.

Keywords: Pathway engineering; Isoprenoids; Toxicity; Mevalonate pathway; *E. coli*

1. Introduction

Recent trends toward the production of complex chemicals and pharmaceuticals in engineered microbes require continual improvements in the design of non-native biosynthetic pathways. Many potentially useful natural products are either too complex to be chemically synthesized or are produced in insufficient quantities in their native host to implement cost-effective crop cultivation and extraction. Alternatively, through metabolic engineering, multi-gene heterologous pathways have been engineered into microorganisms for the production of many important classes of molecules: isoprenoids (Martin et al., 2003; Watts et al., 2005), polyketides (Pfeifer et al., 2001; Peiru et al., 2005), non-ribosomal peptides (Watts et al., 2005), bioplastics (Aldor and Keasling, 2003), and polymer building blocks (Nakamura and Whited, 2003). However, in the course of transferring enzymatic pathways from one organism to another and modulating protein production, the intricate evolved regulation of the pathway is often lost, leading to imbalances in gene expression and enzyme activity. Overexpression of a gene may cause the depletion of precursors or resources necessary for growth and production (Glick, 1995; Jones et al., 2000) or induce a stress response from excessive heterologous protein

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(Goff and Goldberg, 1985; Harcum and Bentley, 1993, 1999), while an imbalance in the total activity of the enzymes can restrict carbon flux. Such a bottleneck in the biosynthetic pathway results in a reduced rate of production (Barbirato et al., 1996; Zhu et al., 2002) and can lead to the accumulation of intermediates and byproducts that inhibit pathway enzymes (Berry et al., 2002) or are cytotoxic (Barbirato et al., 1996; Zhu et al., 2001, 2002).

Engineering metabolic pathways in microbes for the production of scarce therapeutic natural products effective against neglected diseases is an attractive application of this technology. For example, to combat the increasing occurrence of malaria-causing *Plasmodium* strains that are resistant to traditional medications, clinicians have employed the potent anti-malarial drug artemisinin, an isoprenoid natural product extracted from *Artemisia annua*. Unfortunately, artemisinin yields from plant extracts are low, limiting production from Artemisia crops and increasing the cost of artemisinin-based treatments beyond the reach of people in countries most afflicted by malaria. For this reason, we engineered the bacterium *Escherichia coli* as a factory for the synthesis of complex isoprenoids such as artemisinin.

One of the largest obstacles to efficient microbial biosynthesis of isoprenoids is the production of isoprenoid precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Both compounds are produced naturally in *E. coli* through the 1-deoxyxylulose-5-phosphate (DXP) pathway (Rohmer et al., 1993; Lange et al., 2000) (Fig. 1A). Metabolic engineering to overproduce the isoprenoid precursors in *E. coli* has focused on optimizing the native DXP pathway and cellular metabolism to accumulate high levels of carotenoids (Farmer and Liao, 2000; Alper et al., 2005b, 2006; Yuan et al., 2006). Combining up-regulation of the entire DXP pathway with systematic and combinatorial gene knockouts resulted in the production of 0.22 g/L lycopene (Alper et al., 2006). This study focuses on an alternative method to over-produce isoprenoid precursors in *E. coli* by cloning and expressing the high flux, mevalonate-dependent, isoprenoid pathway from *Saccharomyces cerevisiae* (Martin et al., 2003) (Fig. 1A). Using the synthesis of a precursor to the potent anti-malarial artemisinin, amorpha-4,11-diene, as an example, this system demonstrated high isoprenoid production capability, producing 0.48 g/L amorphadiene in two-phase fermentation (Newman et al., 2006). This fermentation titer is similar to the aforementioned studies employing the native DXP pathway and represents a 2400-fold improvement in production over wild-type *E. coli*. However, neither production system currently achieves the isoprenoid titer of 25 g/L estimated to be necessary for providing inexpensive artemisinin to countries most afflicted by malaria (Ro et al., 2006).

In the expression of a multi-gene heterologous pathway, the activity of a single enzyme may be out of balance with that of the other enzymes in the pathway, leading to unbalanced carbon flux and the accumulation of an intermediate. In this paper we describe how balancing carbon flux through the heterologous mevalonate pathway resulted in improved growth and isoprenoid production in *E. coli*. This study demonstrates the importance of retaining balanced flux in a reconstituted heterologous pathway and a methodology for troubleshooting and balancing pathways. Whether the molecule is native or foreign to the host, the unregulated accumulation of any intracellular compound may compromise the viability of the organism or the productivity of the pathway.

2. Methods

2.1. Strains and media

E. coli strains TOP10 and DH10B, both from Invitrogen (Carlsbad, CA), were used for cloning and plasmid construction. In *E. coli* DH10B, the P_{BAD} promoter system suffers from all-or-none induction, in which sub-saturating concentrations of arabinose give rise to subpopulations of cells that are fully induced and un-induced (Khlebnikov et al., 2000). To alleviate this problem for gene titration studies, a DH10B host with regulatable control of P_{BAD} in a homogeneous population of cells was constructed. The chromosomal *araE* gene, was placed under constitutive control of the P_{CP8} promoter and combined with a deletion of *araFGH* following the method of Khlebnikov et al. (2001). The resulting strain, which had a linear response in gene expression as a function of the arabinose concentration across the population, was named DP10 (Table 1).

Media components and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). For cloning and propagation of *E. coli* strains harboring the various recombinant pathways, Luria Broth with Miller's modification (Sigma-Aldrich) was used with appropriate antibiotics for plasmid selection and 0.06% glucose for the repression of P_{BAD} and P_{LAC} promoter systems. Microbial production and gene titration studies were conducted in two different versions of C medium (Helmstetter and Cooper, 1968) as described below. D,L-Mevalonate used for media supplementation was prepared by mixing 1 volume of 2 M D,L-mevalonic acid lactone (Sigma-Aldrich) with 1.02 volumes of 2 M KOH and incubating at 37 °C for 30 min (Campos et al., 2001). Enzymes for molecular biology were purchased from New England Biolabs (Beverly, MA) and Invitrogen.

2.2. Operon and plasmid construction

All plasmids used in this study are listed in Table 1. Construction of plasmids pMevT, pMBIS, pADS, pBAD33MevT and pLac33 were described previously (Martin et al., 2003). The MevT operon was cloned into a variety of expression vectors to determine the effect of plasmid copy number and promoter strength on expression of the cloned pathway (Fig. 1B). The MevT operon was previously cloned into pBAD33 (Guzman et al., 1995), a low copy number vector with the arabinose-inducible