Redirecting carbon flux into malonyl-CoA to improve resveratrol titers: Proof of concept for genetic interventions predicted by OptForce computational framework

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HIGHLIGHTS

- We perform specific genetic alterations to improve malonyl-CoA availability in Escherichia coli.
- Genetic interventions predicted by OptForce increase carbon flux through malonyl-CoA.
- We improve titers of plant natural product, resveratrol by ~60%.
- We find optimal temperature for this fermentation along with the over-expressions.

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ABSTRACT

Malonyl-CoA is the limiting precursor for the overexpression of an array of heterologous pathways in bacteria, such as flavonones, polyketides, microdiesel and poly-unsaturated omega-3 fatty acids. Previously, we have been able to develop a strain with higher carbon flux to acetyl-coA and malonyl-CoA by carrying out genetic interventions predicted by OptForce framework. Here we carried out the same interventions in a resveratrol producing strain and obtained a 60% increase in the yield giving the highest titer of 1.6 g/L obtained in lab scale fermentation without the addition of expensive fatty acid pathway inhibitors such as cerulenin. The positive genetic alterations involved overexpression of pyruvate dehydrogenase multi-enzyme complex (PDH), phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GapA) and deletion of fumarase (FumC). This work presents the development of an alternative source of resveratrol with possible applications in pharmaceutical, nutraceutical and food industry.

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1. Introduction

Malonyl-CoA in one of the universal intermediates formed in both eukaryotes and prokaryotes. It serves as the precursor to the fatty acid synthesis pathway, in turn being an integral part of cell growth as phospholipids are among the building blocks of cell membranes. Over production of certain economically and pharmaceutically important compounds like flavonoids, polyketides, stilbenes and fatty acids necessitates higher amounts of malonyl-CoA since it is the direct precursor of these molecules.

Many attempts have been made previously to engineer strains with improved levels of malonyl-CoA, since it is the precursor to the fatty acid biosynthetic pathway, which in turn is directly related to the cell growth, with its levels tightly regulated inside the cells (Magnuson et al., 1993; Takamura and Nomura, 1988). Because of this strict control on malonyl-CoA concentration inside the cells, a simple overexpression of the acetyl-CoA carboxylase gene does not avail a sufficient improvement in malonyl-CoA levels (Lim et al., 2011). Deletions of enzymes involved in its consumption, such as acetate kinase and alcohol dehydrogenase (Zhaa et al., 2009), have been reported but such deletions have relied mostly on metabolic pathway inspection rather than more systematic exploration. Therefore, it is required to take into consideration the entire metabolic pathway involving malonyl-CoA and its precursors and predict interventions that improve yields without significantly affecting growth, using (for example) stoichiometric-based models. Such modeling is a daunting task considering the myriad of reactions involved in the genome-scale metabolic networks as well as the interconnectivity of the cellular metabolic pathways. For this reason, in order to predict genetic alterations in the strain, many
simplifications and assumptions are made and a manageable number of engineering targets are identified in order to prioritize the implementation of genetic intervention. For example, computational algorithms have been designed to consider the genome-scale metabolic flux measurements that are available to predict targets for engineering an overproduction phenotype. This has led to the identification of up-regulation or deletion gene targets not directly involved in the malonyl-CoA synthesis pathway using OptKnock (Burgard et al., 2003; Pharkya et al., 2003), OptReg (Pharkya and Maranas, 2006), OptORF (Kim and Reed, 2010), Robust Knock (Tepper and Shlomi, 2009) and CIED (Fowler et al., 2009). However, these models are solely based on the utilization of flux balance analysis (FBA). Recently, Xu et al. (2011) reported a 4-fold improvement in the production of the flavonoid molecule naringenin in the host Escherichia coli strain BL21 Star using predictions made by the OptForce (Ranganathan et al., 2010) model. The procedure accounts for a much larger number of pathways than previously considered for predicting relevant genetic interventions. The algorithm employs graph theory for pattern recognition and the information relating to the various reactions that occur inside the cell to map the path of atoms as they are converted from reactants to products in the cell.

Resveratrol, a hydroxylated derivative of stilbenes, is a relatively simple compound produced by several plants via the phenylpropa- noid pathway. It exists in the cis and trans forms, both of which also occur as glucosylated derivatives (glucosides). It is found in grape wines, peanuts, and certain berries, acts as a phytoalexin, and is produced in response to biotic and abiotic stresses such as infections from phytopathogenic micro-organisms and UV-irradiation. Its pharmaco- logical effects gained importance first in 1992 when it was proposed to be responsible for the cardio-protective effects of red wine. It has been shown to have beneficial effects with respect to many chronic human conditions such as cardiovascular disease, ischemic injuries as well as strong anti-oxidant properties. Recently, it was demonstrated that resveratrol also has significant life-span extension properties in various organisms ranging from yeast to certain vertebrates. In 1997 it was reported to inhibit carcinogenesis at various stages (Jang et al., 1997). Being an effective inhibitor of cyclooxygenase activity in vivo (Afaq et al., 2003; Aziz et al., 2005; Khanduja et al., 2005) it has been shown to improve inflammation (Birrell et al., 2005; Chen et al., 2005; Jang et al., 1997; Böhm et al., 2004; Wu et al., 2005a, 2005b). In vitro studies (Howitz et al., 2003) have shown resveratrol to be one of the most potent inducers of SIRT1 (the gene involved in caloric restriction) which in turn slows pace of ageing even though these results have been heavily contested in the recent past. Subsequent work has shown that resveratrol extends the lifespans of Saccharomyces cerevisiae, Caenorhabditis elegans and Drosophila melanogaster (Howitz et al., 2003; Wood et al., 2004). However, the in vivo availability of resveratrol is still under scrutiny, since pharmacokinetic studies indicate that resveratrol is rapidly metabolized in vivo. Further experiments are needed to clear physiological relevance of resveratrol and to determine whether the in vitro effects observed can be reproduced in vivo.

Industrially, resveratrol is still produced from plant extracts. But this process suffers from several limitations like low yields, impurities, and long turn-around time involving the growth period required by the plants. With the development of metabolic engineering and systems biology tools, several successful attempts have been made to produce it in heterologous hosts like E. coli and yeast. These hosts have the advantage of much faster growth rates; ease of genetic manipulation; lack of use of toxic wastes and finally independence from regional variations. As reported previously, titers as high as 1.3 g/L were produced in E. coli (Lim et al., 2011), while yeast has shown the highest production of 391 mg/L (Sydor et al., 2010).

In the present work, we attempted to evaluate the effect of redirecting malonyl-CoA flux towards resveratrol production by applying the OptForce algorithm. By doing so, we were able to reach production titers of 1.6 g/L in shake flask experiments without the need of using expensive inhibitors of fatty acid metabolism.

2. Materials and methods

2.1. Strains and media

Strains DH5α, BW27784 and BW27784 (DE3) (E. coli Genetic Stock Center, New Haven, CT) were used for plasmid cloning and recombinant molecule production. DNA manipulations were performed according to standard recombinant DNA procedures (Sambrook et al., 1989). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. All PCR amplification and cloning reactions were performed using Accuzyme DNA polymerase (Bioline) or Phusion high fidelity DNA polymerase (New England Biolabs). Plasmid DNA was prepared from stock strains using a Zynppy plasmid miniprep kit, while fragment DNA was isolated by gel extraction using a Zymoclean gel DNA recovery kit (Zymo Research). Plasmid-bearing cultures were grown in media containing, when needed, ampicillin (70 µg/mL), kanamycin (40 µg/mL), chloramphenicol (20 µg/mL), and/or streptomycin (40 µg/mL). p-Coumaric acid, the resveratrol standard and cerulein were all purchased from Sigma-Aldrich.

2.2. Computational procedure

The essence of OptForce procedure is to compare and contrast the maximal range of flux variability for a wild-type strain (or a base strain) against the ones consistent with a prespecified overproduction objective. As outlined in our earlier efforts (Ranganathan and Maranas, 2010; Ranganathan et al., 2010), the flux ranges for the wild-type strain can be elucidated by iteratively maximizing and minimizing each flux in the network (Burgard et al., 2003; Mahadevan and Schilling, 2003) subject to constraints pertaining to stoichiometry, uptakes and environmental conditions (i.e. regulation) and metabolic flux analysis (MFA) data. The flux measurements used in this procedure can be either exact values or ranges. By superimposing the flux ranges one-at-a-time, we identify the fluxes that must depart from the original ranges in the face of overproduction (MUST sets). We extend this classification procedure by considering sums and differences of two, three or more fluxes and arrive at a collective set of flux changes that must happen in the network for overproduction. The MUST sets represent the set of all changes that must happen in the network. We subsequently extract the minimal subset(s) of these reactions that suffice in guaranteeing the required bioengineering objective (i.e., FORCE sets).

2.3. Pathway construction

Plasmids pACYCDuet-1 and pCDFDuet-1 were purchased from Novagen, while plasmid pUC18 containing the Arabidopsis thaliana 4CL (At4CL) and the Vitis vinifera stilbene synthase (VWST) genes downstream of the gap promoter (pGAP) was developed in a previous study (Lim et al., 2011).

Two rounds of cloning were used to create pCDM4-pgk–gapA. Genes pgk and gapA were PCR amplified from pCoLA–EcPGK–EcGAPA using primer pairs pgk_NdF/pgk_XhR and gapA_NdF/gapA_XhR, respectively. NdeI and XhoI digested PCR products were inserted into the NdeI and XhoI sites of pCDM4 to give constructs pCDM4-pgk and pCDM4-gapa, respectively. ePath-Brick directed gene assembly was used to assemble these two genes (Xu et al., 2012). Specifically, the small fragment of AvrII
and Sall digested pCDM4-gapA was ligated to the SpeI and Sall digested pCDM4-pgk to give construct pCDM4-pgk–gapA.

Three rounds of cloning were used to create pRSM3-PDH. Genes aceE, aceF and lpdA were PCR amplified from pCoLA-EcpDH using primer pairs AE_AsF/AE_XhR, AF_NdF/AF_XhR and lpd_NdF/lpd_XhR, respectively. Ndel (Asel for aceE) and Xhol digested PCR products were inserted into the Ndel and Xhol sites of pRSM3 to give constructs pRSM3-aceE, pRSM3-aceF and pRSM3-lpdA, respectively. ePathBrick directed gene assembly was used to assemble these three genes (Xu et al., 2012). Specifically, the small fragment of AvrII and Sall digested pRSM3-IdpA was ligated to the SpeI and Sall digested pRSM3-aceF to give construct pRSM3-aceF–aceE–ldpA (all these three genes were connected in pseudo-operon configuration). The final construct pRSM3-aceE–aceF–ldpA was called pRSM3-PDH.

Gene deletions were performed using the red recombinase based chromosomal gene inactivation protocol developed by Datsenko and Wanner (2000). Deletion primers (Table S2) with 40 nucleotide homologous regions were used to create the FRT-flanked kanamycin resistance cassette from pKD4, which was used to delete aceE to give construct pRSM3-aceE–aceF–lpdA (all these three genes were connected in pseudo-operon configuration). The final construct pRSM3-aceE–aceF–ldpA was called pRSM3-PDH.

2.4. Resveratrol fermentations

Fermentation was carried out at two different temperatures, 30 °C and 37 °C, in culture medium consisting of yeast extract M9 medium (YM9) adjusted to pH 7 (M9 salts, 10 g/L yeast extract, 3% glycerol, and 42 g/L MOPS [morpholinepropanesulfonic acid, 99.5% purity from Sigma] along with required antibiotics, to find the optimal expression temperature of the T7 promoter. A pre-inoculum culture was started in the morning and was grown for 6–7 h. The cells were re-suspended in 10 mL of YM9 media, with IPTG, at a starting OD600 of 0.1, and allowed to grow overnight at 37 °C to build biomass. Next day, cells were collected by centrifugation, at room temperature and resuspended in fresh YM9 media containing 100 g/L polyethylene glycol, 1 mM IPTG and 15 mM p-coumaric acid. Production was carried out either at 30 °C or 37 °C. Samples of 100 μL were collected after 6 h. A 100 μL or sometimes 200 μL (for high resveratrol concentration samples) of ethyl acetate containing 1% HCl was added to each sample. The mixture was then vortexed for 30 s and separated by centrifugation before being injected into the HPLC.

2.5. HPLC analysis

For production in BW27784 and BW27784 (De3) strains, 2 μL of the ethyl acetate fraction was injected into the HPLC using a solvent profile with 0.1% (vol/vol) formic acid in acetonitrile (buffer A) and 0.1% (vol/vol) formic acid in water (buffer B) as the mobile phases and a flow rate of 1.0 mL per minute. The mobile-phase composition profile was fixed at 65% buffer B and 35% buffer A for 4 min, with 30 s of post-run time allowed for column equilibration. The resveratrol peak was found to elute at 2.9 min under this solvent profile. Ethyl acetate extraction using twice the volume of culture sampled was done prior to the HPLC injection to prevent polyethylene glycol from the culture medium or protein in the in vitro assay from clotting up the guard column in the HPLC.

3. Results and discussion

3.1. Predictions by OptForce and their genetic significance

The phenylpropanoid pathway involves the phenylalanine ammonia lyase, which converts the amino acid phenylalanine to 4-cinnamic acid, which acts as a substrate for cinnamate 4-hydroxylase, producing 4-coumaric acid. 4-coumaric acid is converted to 4-coumarol-CoA by 4-coumarol-CoA ligase. 4-coumarol-CoA is acted upon by stilbene synthase, which adds three acetyl groups from three malonyl-CoA molecules to form stilbenes by a Claisen condensation reaction. Malonyl-CoA is the starting substrate for the fatty acid biosynthesis pathway intrinsic to E. coli. It is produced by the enzyme acetyl-CoA carboxylase. Its concentration inside the cell is tightly regulated by fatty acyl-ACP and fatty acyl-CoA, since they are involved in the growth rate of the organism and the phospholipid formation.

In previous work, production of resveratrol in E. coli has been optimized to a great extent. The highest production titer of 1.3 g/L was obtained with the A. thaliana 4CL and V. vinifera STS, under a single pGAP promoter in the pUC18 vector, with the high biomass strain BW 27784. Production was further increased to 2.3 g/L upon addition of cerulenin, a free-fatty acid biosynthetic pathway inhibitor, implying that an improvement in production can be obtained by redirecting more malonyl-CoA into the heterologous resveratrol biosynthetic pathway. In the present work, we report overexpressions and deletions aimed to further increase resveratrol yield without the need to supplement with the expensive antibiotic cerulenin. In our previous system we used T7 promoters for overexpression purposes induced by the T7 RNA polymerase, which are not amenable to BW 27784 host strain due to the lack of DE3 genotype. For this reason, the pUC18 vector used to carry the genes VvST5 and At4CL encoding for the resveratrol biosynthetic pathway was transformed into the BW 27784 (DE3) strain, and all overexpressions and deletions were carried out in this strain.

Previously attempted deletions involved a systematic down-regulation of the citric acid cycle of the cell. Since acetyl-CoA, the precursor to malonyl-CoA is consumed by the citric acid cycle, OptForce suggested, the deletion of succinyl-CoA synthetase (SUCOAS) and propionyl-CoA:succinyl-CoA transferase (PPCSCT) that consume coenzyme A as a cofactor leading to the formation of succinyl-CoA. The reaction parameters for these reactions indicate that they consume a considerable amount of coenzyme A. Now, the production of acetyl-CoA from pyruvate (carried out by pyruvate dehydrogenase) requires coenzyme A; therefore, a complete elimination of succinyl-CoA synthetase and propionyl-CoA:succinyl-CoA transferase was predicted. Furthermore, only a down-regulation of malate dehydrogenase (MDH), fumarase (FUM) and aconitase (ACONT a/b) to decrease the uptake of acetyl-CoA into this pathway was predicted (Fig. 1).

Also, the up-regulation of reactions involved in the formation of pyruvate via the glycolysis pathway, glyceraldehyde-3-phosphate dehydrogenase (GAPD) and phosphoglycerate kinase (PGK) was suggested by the model. In addition, the overexpression of pyruvate dehydrogenase (PDH) and acetyl-CoA carboxylase (ACCOAC) were also suggested, so as to increase the conversion of pyruvate produced by the glycolytic up-regulations into acetyl-CoA which in turn gets converted to malonyl-CoA. All of the suggested alterations were individually carried out in the BL21Star strain, and the ones giving highest fold increase in naringenin production were then grouped together in order to find the highest producing genotype. The sucC, fumCfumB deletions gave a ~30% increase in naringenin production, while, the mdh and acna or acnb deletions led to a decrease in the production. Grouping the highest producing deletion strains with the
overexpressions of PGK, PDH and ACC resulted in a 5.6-fold production increase compared to the wild-type.

3.2. Production in genetically altered strains

Based on the previous results, sucC and fumC deletions were carried out in BW27784 (DE3) strain. The fumC deletion gave around 10% increase in production, while the sucC deletion did not alter production. A double deletion strain (ΔfumCΔsucC) could not be created in the lab after several attempts. Therefore, the fumC deletion strain was used to carry out the subsequent overexpressions. Individual overexpressions of PGK, PDH and GAPA did not result in significant increases in the production of resveratrol as compared to the control strain. The combined overexpression of PGK, GAPA and PDH however resulted in a 45% increase in production. Finally, the ACC overexpression was carried out individually with each of the PDH, and PGK–GAPA overexpression strains. The highest increase seen, of approximately 33% with the PGK–GAPA, PDH and ACC overexpression was still less than the one seen with the triple overexpression of PGK, GAPA and PDH (44%). Overall a 60% increase in production was seen after carrying out the fermentation for 24 h, with the highest titers seen at 8 h (Fig. 2).

It was expected that the overexpression of acetyl-CoA carboxylase would improve the production further and facilitate the conversion of the extra acetyl-CoA to malonyl-CoA, but the experimental results showed otherwise. This leads us to believe that the acetyl-CoA carboxylase present in BW27784 (DE3) strain is able to handle the excess acetyl-CoA and convert it into malonyl-CoA. It is also possible that at 1600 mg/L the strain reached its maximum level of production due to biochemical (e.g. unknown feedback inhibition loops) and cell-toxicity reasons. Besides, adding another plasmid carrying the ACC gene might have increased the antibiotic selective pressure on the strain thus lowering its production. Also, interestingly we would have expected to see a higher specific production when the
production was carried out at 30 °C since the T7 promoter is reported to have its optimum activity at this temperature. But growth at 30 °C resulted in a severe lag in biomass generation as compared to growth at 37 °C and a specific production of 35.9 mg/L/OD_{600} at 30 °C was seen, as compared to that of 51.6 mg/L/OD_{600} at 37 °C after 6 h of fermentation (Figs. 3 and 4).
4. Conclusion

Development of mathematical models to predict genetic interventions has been an area of significant advances and continuous improvement over the last few years. With the availability of detailed information about each reaction going on, in a frequently used heterologous host such as *Escherichia coli* and *Saccharomyces cerevisiae*, it is of utmost importance that we are able to utilize this data to predict beneficial genetic alterations in a more systematic and quantitative way. We show here that the predictions made by the OptForce model for improving the availability of malonyl-CoA resulted in substantial improvements of the resveratrol titers, as it was previously demonstrated with another malonyl-CoA-derived polyphenol, naringenin. Additional effort to further optimize expression levels by fine-tuning the promoter strengths used for overexpressing enzymes is currently underway.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ces.2012.10.009.

References


