BioSystems 171 (2018) 10–19

Contents lists available at ScienceDirect

BioSystems

journal homepage: www.elsevier.com/locate/biosystems

Integrated constraints based analysis of an engineered violacein pathway in *Escherichia coli*

Selva Rupa Christinal Immanuel[1], Deepanwita Banerjee[1], Mayooreshwar P. Rajankar[1], Anu Raghunathan*

Chemical Engineering Division, CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune, 411008, India

ARTICLE INFO

Keywords:
Violacein
Escherichia coli
Synthetic biology
Metabolism
Flux balance analysis

ABSTRACT

Strategies towards optimal violacein biosynthesis, a potential drug molecule, need systems level coordination of enzymatic activities of individual genes in a multigene operon vioABCDE. Constraints-based flux balance analysis of an extended *i*AF1260 model (*i*AF1260vio) with a reconstructed violacein module predicted growth and violacein yields in *Escherichia coli* accurately. Shadow price (SP) analysis identified tryptophan metabolism and NADPH as limiting. Increased tryptophan levels in Δ*pgi* & Δ*phe*A were validated using *in silico* gene deletion analysis. Phenotypic phase plane (PhPP) analysis highlighted sensitivity between tryptophan and NADPH for violacein synthesis at molar growth yields. A synthetic VioABCDE operon (SYNO) sequence was designed to maximize Codon Adaptive Index (CAI: 0.9) and tune translation initiation rates (TIR: 2–50 fold higher) in *E. coli*. All pSYN *E. coli* transformants produced higher violacein, with a maximum six-fold increase in yields. The rational design *E. coli:* Δ*pheA* SYN: gave the highest violacein titers (33.8 mg/l). Such integrated approaches targeting multiple molecular hierarchies in the cell can be extended further to increase violacein yields.

1. Introduction

The grand challenge of metabolic engineering lies in the complexity and redundancy of cellular pathways and the evolutionary drive to maximize growth/fitness rather than a forced bioengineering objective. Constraints based flux balance analysis (FBA) of metabolic models has been used to design strains *in silico* that simultaneously maximize fitness and the desired product (Burgard et al., 2003; Ruppin et al., 2010; Varma and Palsson, 1993). These models predict intracellular reaction fluxes and identify strategies for substrate uptake, energy and cofactor balance. Although, these models can drive rational strain design, the predictions of such evolutionary optimality models are more in tune with adapted strains (Ibarra et al., 2002). Metabolic engineering of value added products through synthetic biology strategies to fast forward the adaptive evolution process are becoming more rampant.

When complex pathways are introduced inside the cell, limitations including intermediate toxicity, low enzyme activity, metabolic burden (cofactor imbalance etc.) need to be overcome for high performance. Such bottlenecks can be addressed using pathway engineering that exploits the synergies of synthetic biology, metabolic engineering and systems biology (Nielsen and Keasling, 2011, 2016; Stephanopoulos, 2012; Wu et al., 2016; Yadav et al., 2012). Successful metabolic engineering for platform cell factories to produce a wide range of fuels and chemicals necessitates identifying the sensitivity of product/process to nutrient precursors and cofactors *a priori.* Such complementation supports coupling of cellular objectives of growth and energy to desired bioengineering objectives. Comprehensive computational strain designs for stoichiometric growth-coupling of desired products of central metabolism have been identified through pathway analysis (Klamt and Mahadevan, 2015; Von Kamp and Klamt, 2017).

Violacein is a bacterial bis-indole pigment of commercial interest having antibacterial, antitumoral, antiviral, trypanocidal and antiprotozoan properties (Durán and Menck, 2001; Durán et al., 2007; Ferreira et al., 2004; Queiroz et al., 2012). It is formed by the condensation of two L-tryptophan molecules controlled by the enzymes of a complex biosynthetic pathway (Fig. 1). The impact of the double bonds, conjugation and hydroxyl groups potentially attribute chromophoric properties (Fig. 1B) to final violet colored product violacein of the pathway (Hoshino, 2011). Violacein has been tested to show anti-bacterial (gram positive), antineoplastic and antifungal properties (Durán et al., 2016). Other tryptophan based small molecule therapeutics like rebeccamycin and staurosporine have been reported as important antitumor molecules (Howard-Jones and Walsh, 2006).

The violacein biosynthetic pathway is complex due to a coordinated

* Corresponding author.
E-mail address: anu.raghunathan@ncl.res.in (A. Raghunathan).
[1] Equal contributing author.

https://doi.org/10.1016/j.biosystems.2018.06.002
Received 20 April 2018; Received in revised form 5 June 2018; Accepted 15 June 2018
Available online 03 July 2018

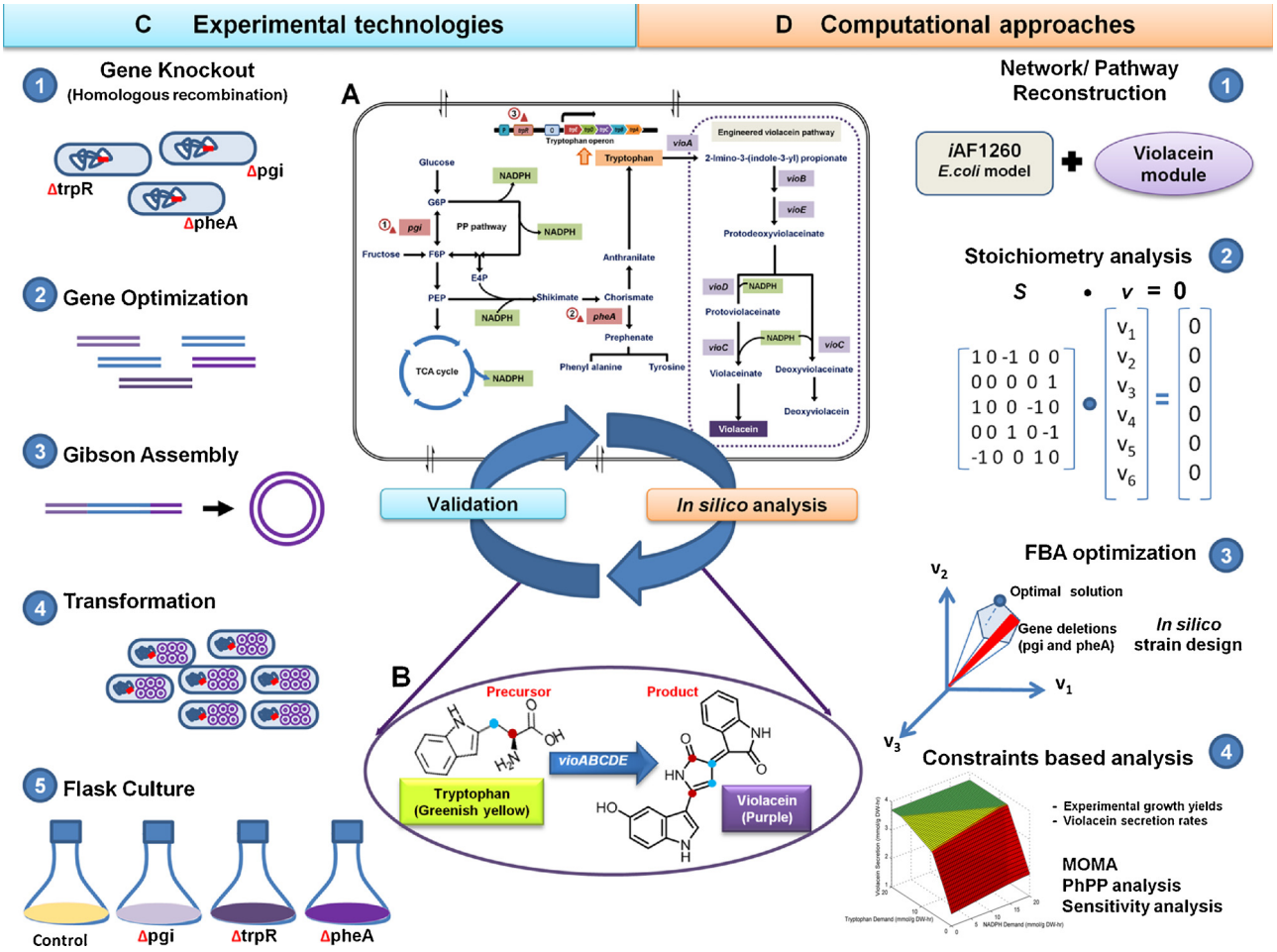

Fig. 1. Integrated approaches to rational strain design and development.
The work-flow of biosynthesis of violacein from tryptophan via a pathway module with coordinated multigene activity (**A**) is depicted. The differential chromophoric properties of the product violacein (λ_{max} = 565 nm), a purple colored pigment, *vis a vis* it's precursor tryptophan (λ_{max} = 280 nm) is potentially through increased conjugation represented (**B**), The right hand panel (**D**) represents the use of computational approaches for metabolic engineering (from network reconstruction to constraints based modeling) while the left hand panel (**C**) represents all experimental technologies involved (from genome engineering to shake flask culture).

five gene operon structure in *Chromobacterium violaceum* (Balibar and Walsh, 2006; Hoshino, 2011). The rate limiting step involves the condensation of two molecules of tryptophan to 2-imino-3-(Indole-3-yl) propionate (*vioA)*. Further steps include conversion to protodeoxy violaceinate by *vioB* and *vioE*, followed by conversion to violacein and deoxyviolacein by catalytic activity of *vioD* and *vioC*. The dynamics of violacein biosynthesis are thus dependent on the coordinated levels of transcripts, proteins, action of promoter and ribosome binding sites (RBS) (Lee et al., 2012; Salis et al., 2009). This necessitates minimization of transcriptional noise (relative stability of transcripts) caused by synthesis and degradation of mRNA molecules and increasing the efficiency of translation of mRNAs into proteins by modulating translation initiation rate (dictated in part by mRNA secondary structures). Translational coupling (TC) significantly modulates translation efficiency of individual genes in a multi-gene operon structure within *E. coli* (Lim et al., 2011; Tian and Salis, 2015).

There have been legacy efforts for violacein production using native producers including *Chromobacterium*, *Duganella* spp. and recombinant *Corynebacterium*, *Citrobacter* and *E. coli* hosts (Table S10). The highest level of violacein attained in native producer *Duganella* spp. B2 is 1.62 g/l. Current efforts on producing violacein in non-native hosts include the extensive testing of the potential of *E. coli* (up to 1.92 g/l with genetic variations) as a platform for production of tryptophan-based therapeutics. The strain designs with *vioABCE* operon from *Chromobacterium* produced violacein up to 70 mg/l in batch processes using arabinose as substrate (Rodrigues et al., 2013). A fed-batch process increased violacein by 10 fold. *Corynebacterium glutamicum* spp. (Sun et al., 2016) and *Citrobacter freundii* (Jiang et al., 2010), classical amino acid producing strains have also been engineered to produced violacein.

In this study, an integrative computational and experimental strategy for strain design and genome engineering of *E. coli* to produce violacein was developed. A violacein biosynthesis module (*vio*) was reconstructed in a previously published genome-scale *E. coli* model, *i*AF1260 (Feist et al., 2007) for the constraints-based flux balance modeling of violacein production. Predicted gene deletions Δ*trpR,* Δ*pheA* and Δ*pgi* that increase the yields of the precursor tryptophan were constructed. The impact of the identified genetic perturbations of the *E.coli* host on violacein production at molar growth yields was delineated experimentally using growth experiments and computationally using FBA. Plasmid constructs pCVW and pSYN based on the Wild-Type VioABCDE operon (WTO) and synthetic operon (SYNO) designs of the vioABCDE operon were expressed in *E. coli* K12 and its derivatives with varying genetic backgrounds. The influence of changing central metabolism via genetic changes in the chassis in the presence and absence of a completely synthetic and wild-type pathway from *Chromobacterium* was tested extensively. To our knowledge this is the first study that combines a synthetic biology approach like operon sequence optimization and constraints-based flux balance modeling for the metabolic engineering of violacein in *E. coli*.