Enhanced Succinic Acid Production in *Escherichia coli* by Model-Guided Metabolic Gene Knockout of pflA Using Glucose Carbon Source

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Abstract

Succinic acid is an important platform and/or commodity or specialty chemical with a broad range of applications. The metabolic role of pyruvate formatelyase A (pflA) in relation to succinate production in *Escherichia coli* under anaerobic conditions from glucose substrate remained largely unspecified. Herein we identified pflA gene for the first time, as a novel gene knockout target for increasing succinate production in *E. coli*. Guided by *E. coli* reconstruction iJO1366, we engineered the *E. coli* host metabolism by deleting the pflA, thereby causing the up-regulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which hypothetically increases the generation of NADH and the pool of phosphoenolpyruvate (PEP) in the central carbon metabolism, required for succinate production. This strategy produced succinic acid that is 4.78 fold (0.28g l-1 in 1 day) from glucose substrate. This work elucidates for the first time that pflA is a novel gene deletion target for increasing succinic acid production in *E. coli* under anaerobic conditions. In addition, these results highlight the power of metabolic model in identifying novel gene deletion target and ultimately driving novel biological discovery.

Keywords: *Escherichia coli* genome-scale model; Metabolic gene knockout prediction; Pyruvate formatelyase (pflA); Glucose carbon source; Enhanced succinate production

Introduction

Microbial fermentation for succinic acid production has been pursued in recent time because it is considered cheaper and environmentally friendly approach than its petroleum based chemical production from maleic anhydride [1]. Recently, several bacteria, such as Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes and Mannheimia succiniciproducens have been established to produce succinic acid as a major fermentation product [2]. However, these strains require complex organic nutrients that increase the costs of productions, purifications and the waste disposal which ultimately add to process costs and complexity [2,3]. *Escherichia coli* have been known to naturally carry out mixed acid fermentation with succinic acid as a minor fermentation product among others. As a specialty and/or commodity chemical, succinic acid has invaluable applications, such as a precursor for various chemicals, including green solvents and biodegradable plastics, it can also be used as an iron chelator and a supplement to many foods and pharmaceuticals [1,2]. Succinic acid has also been listed by the U.S. Department of Energy (DOE) among the 12 top bio-based building block chemicals that can be produced by microbial fermentation [4,5]. Several numbers of metabolically engineered *E. coli* strains were constructed, with or without foreign genes for enhanced succinate production using glucose substrate [2]. While others, used mineral salt medium to produce succinate in metabolically engineered *E. coli* strains by knocking out pyruvate formatelyase B (pflB) [3]. pflB was previously designated as a formate acetyltransferase I, which its deletion under anaerobic conditions blocks formate formation and increase succinate production [3,6]. The disruption of pyruvate formatelyase (pfl) was established to increase D-lactate production in *E. coli* under micro-aerobic conditions [7]. The deletion of pflA for increasing succinic acid production has not yet been elucidated.
The proliferation of *E. coli* genome scale models (GEM) [8,9] have facilitated the application of systems metabolic engineering to increase the production of desired compounds. One of the application of *E. coli* GEM is in metabolic engineering interventions and targeted biological discovery among others [10]. Although recently a number of studies have shown that GEMs of *E. coli* can be deployed for metabolic gene knockouts in increasing succinic acid production, only few studies reported the use of *E. coli* GEM to guide metabolic engineering [11]. The use of *E. coli* genome scale metabolic model to guide future experimental studies would offer considerable help in reducing the time and costs of a targeted biological discovery. Direct experimental trial and error approach was employed to increase D-lactate production in *E. coli*, following the metabolic gene knockout of the entire pyruvate formate-lyase (pflA, pflB, pflC and pflD) [7]. These deletions, particularly of pflA and pflB were established to cause up the regulations of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PYK) glycolytic enzymes, thereby generating NADH that facilitated increase purely D-lactate production in *E. coli* [7]. But the specific role of pflA when deleted for increasing succinic acid production has not been reported. The current study hypothesizes that pyruvate formate-lyase activating enzyme 1 (encoded by pflA) could be a novel gene knockout target for increasing succinate production in *E. coli*. To facilitate and expedite our efforts, we leveraged predictive computational modeling of metabolism and model-guided analysis of experimental data. I applied constraint-based metabolic modeling by deleting the pflA gene using both the substrates to increase succinate production in *E. coli*. This is because researchers have reported the successful application of metabolic models of *E. coli* to engineer strains that produce succinic acid [1,11]. Herein we report for the first time the model-guided identification of pflA as a novel gene deletion target for increasing succinate acid production in *E. coli* as initially hypothesized. An accurate *E. coli* GEM [9] and Minimization of Metabolic Adjustment (MOMA) algorithm [12] in the OptFlux software platform [13] were utilized for the prediction of the target, and subsequently confirmed experimentally. It is worth mentioning that the current study achieved construction of an *E. coli* mutant strain designated as BSM3 with succinic acid production titer that is 4.7 fold (0.28g l⁻¹) in 1 day from glucose substrate. This study informs other studies that pflA is a novel target that can be deleted to increase succinic acid production in *E. coli* and probably beyond.

**Materials and Methods**

**In silico analysis of gene knockout**

*Escherichia coli* genome scale stoichiometric model iJO1366 [9] was employed for the in silico simulation of gene deletion by using Minimization of Metabolic Adjustment (MOMA) algorithm [12] with OptFlux software platform [13]. The *E. coli* iJO1366 model has been tested and proven to be predictive for computations of growth rates and metabolite excretion rates from a range of substrates and genetic conditions [9,14]. MOMA was described as a flux based analysis technique that employs quadratic programming to search for the nearest point in the feasible solution space of the mutant model in relations to its wild-type optimal point feasible solution space [12]. The OptFlux software platform is an in silico metabolic engineering (ME) platform that was implemented using the Java programming, which contains MOMA as a simulation algorithm. Flux balance analysis (FBA) was used for all phenotype simulations. All the simulation of the mutant and the wild-type models were performed using the OptFlux software version 3.07 Glucose was used as a solitary carbon source under anaerobic conditions. The substrate uptake rate was constrained to a maximum of 18.5mmol·gDW⁻¹·h⁻¹ whereas the corresponding oxygen uptake rate was set to zero, as the environmental conditions are anaerobic. These values were selected based on closely established experimental observations on aerobic and anaerobic growth in *E. coli* [15,16].

**Bacteria and plasmid**

*E. coli* JM109 (F(-) traD36, proAB+ lacIq, D (lacZ) M15) endA1 recA1 hsdR17 (rK· StrR) mcrA ApE44I gmrA96 relA1 D (lacproAB) thi- was used for maintenance of the pkD4 and pkD46 plasmids. The plasmids were used strictly following the method described previously [17]. The plasmid pkD4 was extracted from *E. coli* JM109 using the Quick-prepMiniprep kit according to the manufacturer’s specifications.

**Media chemicals and other reagents**

*E. coli* cells used in this study were grown in LB medium containing 0.5% yeast extract (Difco), 0.5%NaCl and 1% Bactotryptone (Difco) without or with antibiotics at the concentrations of 100µg/ml ampicillin and 30µg/ml of Kanamycin. L-arabinose, and glucose were obtained from Sigma Aldrich. KAPA HiFiHotstart Ready Mix (2X) was from KAPA BIOSYSTEMS. Agarose was purchased from (Sigma Aldrich).

**PCR primers**

The *E. coli* pflA gene sequence was used to design forward and reverse primers with pkD4 template plasmid sequence. The primers had 50-nt 5’ extension including the gene initiation codon (H1) and 20-nt sequence (p1) as described previously [17,18]. Table 1 gives the details of the primers used in this study.

**Generation of PCR fragments**

PCR reactions were carried out in an Eppendorf thermo cycler using 25µl reactions containing 12.5µl of KAPA HiFiHotstart Ready Mix (2X), 1µl of pkD4 template DNA, 1.0µl of each primer. Reactions were performed for 30 cycles: 95 °C for 3min, 98 °C for 20 secs, 55 °C for 15 sec, 72 °C for 1:30 sec, 72 °C for 60 sec and cooling at 4 °C . PCR products were purified using SV gel and PCR clean up system (Promega, USA), according to the manufacturer’s protocol. Then, the PCR products obtained were analyzed by 1% agarose gel-electrophoresis using 1X Tris-acetate buffer.

**Electroporation and mutant selection**

*E. coli* JM109 harboring the λ-Red helper plasmid pkD46 was grown in 100ml of LB medium with amplicillin and 1mM L-Arab.
inose at 30 °C to an OD 600 of 0.3. Competent cells for electroporation were prepared as described previously [19]. A 1.0µl (400ng) aliquot of the PCR fragment was mixed with 5µl of competent cell in an ice-cold Eppendorf electroporation cuvette (0.2cm). Electroporation was performed at 2.5KV with 2mF and 600Ω and was followed by immediate addition of 1ml of SOC medium (0.5% yeast extract (Difco), 2% Bactotryptone (Difco), 2.5mMKCl, 10mM NaCl, 10mM MgCl2, 10mM MgSO4 and 20mM glucose) with 1mM L-arabinose. The SOC medium mixed with the electroporated cells was incubated for 2 hours at 37 °C. Selection of kan4 transformant was followed immediately by spreading one-tenth portion of the electroporated cells onto kanamycin agar plates as described by Baba and colleagues [18]. To test for accurate mutational inactivation or correct chromosomal structure, 20µl PCR verification method was conducted with kanamycin specific primers K1 and K2 as described earlier [17].

Table 1: Escherichia coli K12 strains, plasmids and primers used in this study.

| E. coli Strains | Relevant Characteristics | Sources |
|-----------------|--------------------------|---------|
| JM109           | Wild-type (Ft (traD36, proAB+, lacIq, D (lacZ) M15) endA1 recA1 hsdR17 (rk-, mk+) mcrAsup44 |- gyrA96
|                 | mlA1 D (lacpro AB) thi3) | Lab collection |
| BMS3           | ΔpflA: FRT-kan-FRT       | This study |
| plasmids       |                          | [17] |
| pKD4           | bla FRT-kan-FRT (Temlate plasmid for FRT-flanked kanamycin resistant gene; AmpR, KmR) | |
| pKD46          | blay βexo (Red recombinase helper plasmid), temperature-condition replicon (Red recombinase expression vector; AmpR) | [17] |
| Primers        |                          | This study |
| pflA_F         | 5’-TTAGACATTGCTTGACATCCATTTTCAACAGGTTTTGCTGTGGCAATTAATCTTGAGGAGCGTGTCTCC-3’ | |
| pflA_R         | 5’-ATTCGACTTTGCTTACGACATCTTCTTGGAGGAGACATATCCTATCCTGGAT-3’ | This study |

Anaerobic fermentation

Bacterial cells starter culture was made by growing the cells in 10ml LB medium with shaking at 200rpm at a temperature of 37 °C. One milliliter of seed culture was inoculated to a 125ml butyl rubber stoppered serum vial, which contained 100ml of fermentation media as described by Lee and colleagues [1]. The fermentation media used contained the following ingredients (per liter): yeast extract=5g; glucose=9g (50mM); NaHCO3 =10g; Na2HPO4 =8.5g; KH2PO4 =15.5g (pH=7.0). Anaerobic conditioning was established by filling the headspace with N2 and was followed immediately by spreading one-tenth portion of the electroporated cells onto kanamycin agar plates as described by Baba and colleagues [18]. To test for accurate mutational inactivation or correct chromosomal structure, 20µl PCR verification method was conducted with kanamycin specific primers K1 and K2 as described earlier [17].

Results and Discussion

Escherichia coli genome-scale metabolic model could help in identifying novel gene deletontargets for increasing succinic acid production. In this study, we initially hypothesized that the deletion of pflA gene in E. coli could increase succinic acid production under anaerobic condition using glucose as substrate. The predicted results obtained with the E. coli GEM using glucose substrate shows decrease in succinate production (95% of the wild-type model) following the deletion of pflA (Table2), but the experimental validation with the same substrate proves otherwise, which is nearly 4.7 fold in 1 day (0.28gl-1) and 3.2 fold in 3 days (0.30g l-1) and their corresponding parent strains produced only 0.058g l-1 and 0.096g l-1 respectively (Table 3). On one hand, the hypothesis that the pflA gene deletion could increase succinic acid production in E. coli under anaerobic condition have been experimentally validated while on the other hand inconsistencies exists in model’s predictions results relative to the experimental outcomes. It was reported previously [7] that pyruvate is mainly cleaved via pyruvate formate lyase (pfl) to form formate and acetyl-coA [19]. The specific deletion of pflA is not clearly specified in relation to succinate production in E. coli under anaerobic conditions, but it was established to cause up regulation of GAPDH and PYK when compared to their parent strains [7]. On the basis of these findings, the plausible hypothetical mechanism for the increased succinate production in strain BMS3 (ΔpflA) could be theoretically attributed to the up regulation of GAPDH and PYK (Figure 1). The up regulation of PYK was previously described as a gluconogenic process using NADH-linked malic enzyme that increases succinate production in E. coli [2,20].
Table 2: *In silico* prediction results.

| E. coli Models Used | Substrate | Deleted Gene  | Growth Rates (h⁻¹) | Succinate (mmol gDW⁻¹ h⁻¹) | % Succinate | Acetate (mmol gDW⁻¹ h⁻¹) | Ethanol (mmol gDW⁻¹ h⁻¹) | Formate (mmol gDW⁻¹ h⁻¹) |
|---------------------|-----------|--------------|--------------------|----------------------------|-------------|------------------------|--------------------------|--------------------------|
| Orth model (WT)     | glucose   | -            | 0.47772028         | 0.153                      | 100         | 15.00003               | 14.7746                  | 31.5658                  |
| BMS3                | glucose   | pflA/b0902   | 0.36140148         | 0.146                      | 95.42       | 15.06427               | 14.77792                 | 31.44083                 |

Figure 1: Metabolic pathways involved in the anaerobic utilization of glucose in *E. Coli* [2,3] and metabolic pyruvate formate-lyase (pflA) gene knockout strategy employed for producing succinate in this study.

The anaerobic conditions established during the fermentative production of succinate in BMS3 strain, might have led to stepping up of glycolysis, and ATP may have been generated via substrate level phosphorylation. This phenomenon coupled with pflAgene knockout might have theoretically lead to the generation of additional NADH through the up regulation of GAPDH which resulted in excess NADH/NAD⁺ ratio in BSM3 mutant strain. Correspondingly, the succinate production increase was achieved to meet the requirements of redox balance and the energy production through glycolysis. The production of succinate in *E. coli* using glucose under anaerobic condition was previously established to consume 2 molecules of NADH per succinate produced [21]. The enzymes in bold (GAPDH and PYK) were up regulated following the pflA gene, which could have been responsible for increased succinate production. Relevant genes and enzymes involved in succinate productions are shown in italics. Broken line indicate additional CO₂ generated following the oxidation of formate to CO₂ and H₂ by formate hydrogen lyase (FHL). The additional CO₂ generated may have contributed to additional CO₂ fixation by ppc for the PEP conversion to OAA, and step wisely converted to succinate. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PYK, pyruvate kinase; PEP , phosphoenolpyruvate; ppc, phosphoenolpyruvate carboxylase; mdh, malate dehydrogenase; fumABC, fumarate hydratases; frdABCD, fumarate reductases

The single deletion of the pflA gene might have increases the NADH pool, because lactate dehydrogenase (ldhA) and alcohol dehydrogenase (adhE) genes were not deleted in our mutant strain BMS3, to primarily show that the succinate production increase is caused by pflA gene knockout. Correspondingly, fermentative profile of the mutant strain BMS3 indicated a clear increase in succinate, lactate and ethanol production from the wild-type using glucose substrate (Table 3).
Table 3: Fermentative succinate production profile from glucose by engineered E. coli strain BSM3 (ΔpflA) with its wild-type strain used in this study*

| Strains | Fermentation Time (days) | OD600 | Glucose Consumption | Succinate | Lactate | Formate | Acetate | Ethanol | Succinate Yield (g g⁻¹ Glucose) | Fold Change |
|---------|-------------------------|-------|---------------------|-----------|---------|---------|---------|--------|--------------------------|-------------|
| WT      | 1                       | 0.576±0.012 | 8.219±0.09 | 0.0580±0.021 | 4.5382±0.011 | 4.411±0.01 | 6.0614±0.002 | 2.259±0.130 | 0.007154 | 1 |
| BMS3    | 1                       | 0.794±0.004 | 8.993±0.00 | 0.2809±0.00 | 9.2563±0.511 | 3.3589±0.01 | 4.7569±0.01 | 2.3604±0.034 | 0.03123 | 4.78 |
| WT      | 2                       | 0.511±0.002 | 8.993±0.02 | 0.159±0.001 | 5.543±0.701 | 4.331±0.01 | 6.198±0.002 | 3.0479±0.15 | 0.0781 | 1 |
| BMS3    | 2                       | 0.772±0.012 | 8.9902±0.01 | 0.3422±0.002 | 9.0592±0.055 | 3.5893±0.07 | 4.9166±0.007 | 2.9669±0.002 | 0.03867 | 2.15 |
| WT      | 3                       | 0.187±0.002 | 8.944±0.01 | 0.0962±0.002 | 5.4515±0.011 | 4.3218±0.004 | 5.9112±0.03 | 2.6667±0.131 | 0.01069 | 1 |
| BMS3    | 3                       | 0.488±0.002 | 8.9935±0.00 | 0.30809±0.00 | 9.3678±0.211 | 3.6717±0.00 | 4.7532±0.006 | 2.6965±0.08 | 0.03426 | 3.2 |

*Data represent the averages of three samples (mean ± standard deviations) taken from days of anaerobic fermentation cultures supplemented with 9 g l⁻¹ of glucose unless otherwise specified.

*Anaerobic vial fermentation on 9 g l⁻¹ initial glucose for 1 to 3 days

*Calculated by subtracting the initial glucose concentration from the residual glucose concentration

*Calculated as (g l⁻¹ of succinate produced) / (g l⁻¹ of glucose consumed)

*Calculated as succinate titer in mutant / succinate titer in the wild-type.

The increase in ethanol and lactate production in this study would not have been possible in the cell without increase in NADH pool, because production of lactate and ethanol are established NADH linked phenomenon [22-24]. Therefore, the additional NADH generated has been used in increasing succinate production in the mutant strain BMS3. This study clearly establishes that single pflA gene knockout is solely responsible for the increased in succinate production under anaerobic conditions in E. coli from glucose substrate. Another interesting reason that could be possible for increasing succinate production in the mutant strain BMS3 with the glucose substrate is the activities of phosphoenolpyruvate carboxylase (PPC) and acetate kinase (ACK). These two enzymes were previously reported to have increased activities following the deletion of pflA [7]. The PPC is the first enzyme for succinate production in E. coli, therefore, based on this enzyme activity, significant succinate production is achieved in E. coli using glucose under anaerobic conditions. In a similar study reported for purely D-lactate production under micro-aerobic conditions, pflA gene knockout in E. coli did not increases succinate production because of CO₂ and PEP shortage, as the condition for D-lactate production in E. coli was micro-aerobic (limited amount of O₂ enters the system) [7].

In contrast, the mutant strain BSM3 achieved increase in succinate production with the same pflA gene knockout, because the fermentative condition employed in this study is completely anaerobic, supplying additional CO₂ for PPC and increasing the pool of PEP by the up-regulation of GAPDH (Figure 1). The blocking of the entire pyruvate assimilation pathway under micro-aerobic and anaerobic conditions by inactivating the pflABCd, could cause the shortage of acetyl-CoA (AcCoA) in E. coli [7]. Although, under fermentative condition, pflB is the predominant route for pyruvate conversion to acetyl-CoA synthesis [25]. pflB was established to be responsible for formate formation, which can be subsequently cleaved to CO₂ and H₂ by formate hydrogen lyase (fdhF/hycB-1) [26] (Figure 1). The reason why we decided not to delete pflB in this study is because we need formate generation which could be subsequently converted to CO₂ and H₂ and additional CO₂ is required for the efficient functioning of PPC to convert PEP to OAA, which could ultimately be used for succinate production. Reduced formation of formate and acetate on glucose substrate was demonstrated by our mutant strain BMS3 (Table 3), because of the deletion of pflA gene and impaired CO₂ production from glucose by the up-regulation of GAPDH [7].
of ATP by utilizing acetate to produce intracellular AcCoA [7]. Our mutant strain BMS3 is deficient in pflA, therefore AcCoA formation via pflA will be minimized, ACK-Pta reactions could occur in the direction favoring AcCoA formation and utilizing acetate and ATP for biomass formation and energy maintenance. This could be the plausible reason why even after the deletion of the pflAgene, yet acetate and formate were produced as fermentation end product in our mutant strain BMS3 (Table 3). Taken together, the model-guided deletion of the pflA gene in E. coli for succinate production from glucose substrate described in this study, is a step forward towards understanding the metabolic role of this deletion and suggests that model can drive novel biological discovery.

The inconsistencies in the results for succinate predicted flux and observed experimental measurements reported in this study represent true biological gaps (incomplete knowledge gaps) in the reconstruction iJO1366 [27]. In addition, models contains missing regulatory processes, and thus could open up further opportunities for novel biological discovery [28-30] on the missing pflA gene function in relation to E. coli metabolism and succinate production under anaerobic conditions. In addition, the current study also clearly established that novel gene deletion targets could beidentified by combining expert knowledge, model-guided and/or systems based metabolic engineering strategies for microbial strain improvement. This strategy could also offer a considerable biological insight for strain improvement for the production of value-added compounds, such as succinate, xylitol, ethanol etc., from renewable feedstock such as glucose.

**Conclusion**

The current study hypothesizes that the deletion of pflA in *Escherichia coli* could increase succinic acid production using glucose carbon source. This hypothesis was predicted using *E. coli* GEM and later experimentally confirmed to have increased succinic acid production from glucose carbon source, suggesting that pflA could be considered as a novel gene deletion target that could increases succinic acid production in *E. coli*, and could ultimately guide future metabolic engineering strategies for increasing the acid production and/or any other chemical that requires additional NADH for its production in *E. coli* and beyond.

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