行政院原子能委員會

委託研究計畫研究報告

纖非糧生質精煉催化轉換技術及應用評估研究

Assessment for non-crop based biorefining technology and application

黃稟傑[1], 蔡佳茵[1,2], 陳宥安[1], 蘭宜錚[1]

[1]國立交通大學生物科技學系, [2]國立交通大學分子醫學與生物工程所

計畫編號：1062001INER007

受委託機關(構)：國立交通大學

計畫主持人：蘭宜錚

聯絡電話：03-571-2121 ext 59719

E-mail address：ethanilan@nctu.edu.tw

核研所聯絡人員：涂瑋霖

報告日期： 2017 年 11 月 26 日

目　錄

中文摘要

在全球人口的增加下，傳統的糖類生物資源逐漸不適用於發酵生產工業用化學品與生質燃油。因此非糧食類的生物資源將成為合成生質化學的重要一項技術。 近年來，利用甲醇合成其他長碳鏈化合物得到相當的重視，主要因為甲醇是非糧食類資源並可同時由較低成本的石化資源與生質物取得。 另外，因應未來頁岩天然氣的量產，單一碳源(C1)的利用也成了一項重要的研發方向，並且，甲醇也是目前較可行的二氧化碳再利用產物之一，但甲醇價格過於低廉，因此，開發甲醇高值化技術變為重要研究方向。本計畫利用天然甲基營養生物進行代謝工程改造使其轉換甲醇成琥珀酸。 琥珀酸(丁二酸)是眾多附加價值化學品(value added chemicals)中極重要的一個，目前廣泛用於食品工業、藥物賦形劑、以及塑膠合成，但更重要的是琥珀酸能進一步衍生成其它重要工業用C4化學品例如：1,4-丁二醇、丁二烯、順丁烯二酸酐、四氫呋喃等。

Bacillus methanolicus 是革蘭氏陽性的甲基營養生物，並已有文獻利用 *B. methanolicus* 進行胺基酸和成。我們發現在 *B. methanolicus* 中表現 α-ketoglutarate decarboxylase 與 succinate semialdehyde dehydrogenase 使得 *B. methanolicus* 產約 10 mg/L 的琥珀酸。之後我們又以在培養基中添加琥珀酸之前驅物 α-ketoglutarate 發現琥珀酸產率可提高至約 100 mg/L，另以添加 glutamate 可將琥珀酸產率可提高至約 160 mg/L。但由於利用過量表現 TCA 酵素基因對於琥珀酸產量沒有明顯提升，我們認為 α-ketoglutarate decarboxylase 與 succinate semialdehyde dehydrogenase 組成之途徑較不適合琥珀酸生產，本研究進行另一琥珀酸生產途徑 Glyoxylate shunt，利用 *B. methanolicus* 原生的 isocitrate lyase 和 malate synthase 在 *B. methanolicus* 進行過量表現，雖過量表現酵素活性明顯提升，但琥珀酸產量仍未提高。透過分別過量表現兩種琥珀酸合成代謝途徑，*B. methanolicus* 無法提升琥珀酸的產出。我們在 *B. methanolicus* 中對前半部的檸檬酸循環進過量表現，希望將碳通量導入琥珀酸合成，但就結果顯示，亦無效於提升琥珀酸產量，同時谷氨酸也未提升，因此，我們認為目前琥珀酸生產最有可能的限制原因主要為原生 succinate dehydrogenase 活性過高，導致合成的琥珀酸快速被降解。就目前結果來看，利用 *B. methanolicus* 雖可行，但仍需解決上述兩項挑戰並提高產量。

本研究另評估 *Methylobacterium extorquens* AM1 對甲醇使用之能力以及分析其對於生產琥珀酸的潛力。*M. extorquens* AM1 於滋養培養基中生長雖快，但會形成非融性顆粒，導致在養殖與對其做基因改造的困難，本研究分析其培養基配方，並達到以滋養培養基與基礎培養基以１：１為最佳生長條件。為可使 M. extorquens 生產琥珀酸，我們著手開發基因抑制技術，而為此我們在本研究中測試出多個啟動子，而後持續進一步開發基因抑制方法。本研究結果顯示，雖使用甲醇生產琥珀酸可行，但須要開發更多基因編輯與調控工具，以利後續碳通量的調控與提升。

英文摘要

As world population increases, traditional bioresources such as sugars are less suitable for use in the production of chemicals and fuels. As an alternative, methanol is an attractive non-food feedstock for the production of value added chemicals. Methanol is available at low price from petrochemical resources and can be derived from biomass. Furthermore, with the increase availability of natural gas resources, one carbon (C1) utilization becomes an important direction for technological development. Methanol has also been viewed as a potential CO_2 storage compound as it can be synthesized via CO_2 with H_2. However, as methanol is a cheap product, its upgrading into higher value product is an important direction. Naturally, methylotrophic microorganisms grow utilizing methanol. It is therefore an appealing direction to metabolically engineer methylotrophic microorganisms for converting methanol to value added products. In this study, we engineered a model methylotrophic gram-positive bacteria *Bacillus methanolicus* for the production of succinate from methanol. Succinate is one of the top most value added chemicals currently used as a food additive, pharmaceutical stabilizer, and the synthesis of synthetic rubber. Derivatives of succinate including 1,4-butanediol, 1,3-butadiene, maleic anhydride, and tetrahydrofuran are common industrial chemical feedstocks with high volume demands.

We observed that overexpression of α-ketoglutarate decarboxylase and succinate semialdehyde dehydrogenase enabled small amounts of succinate production from *B. methanolicus* PB1 of roughly 10 mg/L. We further observed a sharp increase in succinate secretion upon supplementing α-ketoglutarate in the culture medium to about 100 mg/L, indicated that α-ketoglutarate flux may be a potential limiting step to succinate production. Upon feeding of glutamate, the succinate titer further increased to 160 mg/L, suggesting the importance of increasing carbon flux to α-ketoglutarate. However, the overexpression of TCA cycle genes was not effective for increase succinate titer. As a result, we turned to another succinate-producing pathway, the glyoxylate shunt. We overexpressed the natural B. methanolicus isocitrate lyase and malate synthase. While the enzyme activity was confirmed, the succinate production did not increase. Therefore, we think that the difficulty of using *B. methanolicus* for succinate production is primarily due native succinate dehydrogenase is highly active and metabolizes succinate rapidly. Although succinate production from methanol using these engineered methylotrophs is possible, the above major challenges must be met before this production is considered.

In this work, we also investigated *Methylbacterium extorquens* AM1 for its growth and utilization of methanol, as well as its potential for succinate production. Although *M. extorquens* grow fast under complex media, insoluble pellets form, hampering further cultivation and metabolic engineering. Through our study, we identified a suitable growth media that consists of half complex-media and half minimal media for AM1. We also aimed to develop gene inhibition tools for down-regulation of succinate dehydrogenase, which is present in the ethylmalonyl-CoA cycle and used by AM1. Towards this goal, this work characterized nine different promoters and is in the progress of developing gene inhibition tools. Overall, this work showed that succinate production from methanol is possible. However, it is currently limited by our available genetic tools to better develop our methanol microbial cell factories.

壹、計畫緣起與目的

To circumvent the dependence of petroleum, alternative methods for producing petrochemicals or their replacements are of utmost importance in both academics and the industry. Recently, various biochemical productions including that of ethanol, 1,3-propanediol, succinate, etc. have been successful in commercialization. While successful, these productions are typically sugar based. With the continuing increase of world population, food based resources, including sugars, are becoming less suitable for production of industrial chemicals due to competence with food. Therefore, as a result, recent attention has been shifted to non-food based resources such as lignocellulosic biomass and various wastes. In particular, methanol is an attractive non-food feedstock can potentially be used to produce value-added products. Methanol is available at low price from petrochemical resources, in particular from nature gas, and can also be derived from biomass. Naturally, methane and synthesis gas (syngas) is produced from anaerobic digest in waste treatment and landfills. Subsequently, syngas can be converted to methanol. Furthermore, with the increase availability of shale natural gas resources, one carbon (C1) utilization becomes an important direction for technological development. In addition, currently methanol is one of the best compound for CO_2 storage as it can be synthesized from CO_2 and H_2. However, as methanol is a cheap product, its upgrading into higher value product is an important direction. Naturally, methylotrophic microorganisms grow utilizing methanol. It is therefore an appealing direction to metabolically engineer methylotrophic microorganisms for converting methanol to value added products.

一、Importance and application of succinic acid

Succinic acid (Succinate) is a four carbon (C4) dicarboxylic acid which was listed as one of the top value added chemicals from biomass mandated by the U.S. Department of Energy. Succinate is currently produced primarily through catalytic hydrogenation of petrochemically derived maleic acid or maleic anhydride (1) with annual market of 30,000 to 50,000 tons. The current existing markets for succinate are in the detergent/surfactant, ion chelator, food, and the pharmaceutical markets (1). In

addition to the existing markets, succinate can also be derived into other commodity chemicals (Figure 1) such as 1,4-butanediol (1,4-BDO), gamma-butyrolactone (GBL), and tetrahydrofuran (THF). These chemicals are currently produced from petroleum with annual production of multi-million tons and used for polymer synthesis and as solvents in large scales. In particular, 1,4-BDO and succinate can be used to produce biodegradable poly(butylene succinate), which has better properties and greater stability than polymers produced from 1,2-propanediol or ethylene glycol, and other polyester polyols, plasticizers and polyurethanes. 1,4-BDO can also be dehydrated into butadiene, which is used to make synthetic rubber on a large scale. Recently, many multinational corporations, including BioAmber, BASF, Myriant, Mitsubishi, and DuPont, are commercializing biosuccinate and succinate based polymer production from sugar, indicating the commercialization feasibility of biosuccinate. Succinate is both a key intermediate of the TCA cycle and a fermentative product naturally present in the biosphere. As such, succinate is relatively less toxic to microbes, which may facilitate its higher productivity than other non-natural products. Biosuccinate production from sugars has been studied over more than a decade. Various heterotrophs have been either metabolically engineered (2-6) or selected (7) to produce succinate at various titers. Furthermore, succinate production from hemicellulose, a key component of plant biomass, has been demonstrated with titer up to 14.4 g/L in batch fermentation using engineered *E. coli*. Therefore, methanol-based succinate production may directly be plugged into the current biosuccinate production and market.

二、Methylotrophs

Methylotrophic microorganisms are capable of utilizing methanol as the sole carbon source for growth. In the past decade, significant interest was paid to developing methanol-based amino acid producers (Table 1). In particular, both wild type and mutant *B. methanolicus* strains were isolated for production of lysine (8) and glutamate (9). Additionally, *Methylobacterium extorquens* and mutant *Methylobacillus glycogenes* were isolated for production of serine (10) and threonine (11), respectively, from methanol. As methanol-based chemical production has recently drawn attention, some methylotrophs have been engineered

to produce bioproducts including n-butanol (12), Cadaverine (13), mesaconate (14), and humulene (15). However, to date, there is no report on production of succinic acid from methanol.

B. methanolicus assimilate methanol into its metabolism through the ribulose monophosphate (RuMP) pathway. Methanol is first oxidized to formaldehyde using a NAD-dependent methanol dehydrogenase (Mdh). *B. methanolicus* MGA3 genome and native plasmid pBM19 encode for three Mdhs. In particular, it has been shown that the methylotrophy of *B. methanolicus* is dependent on the plasmid pBM19 (16) which contains the most active Mdh and core set of the genes required for RuMP pathway. While the standard Gibbs free energy change for the oxidation of methanol is +34.2 kJ/mol, *B. methanolicus* drives this reaction through rapid reoxidation of NADH through respiration, thus requiring ample amount of oxygen for optimal growth on methanol. Similarly, B. methanolicus PB1 harbors a natural plasmid pBM20 which is equivalent to MGA3's pBM19

三、Bio-Succinic acid

There are many natural metabolic pathways leading to the synthesis of succinate. Succinate is a natural fermentation product from some bacteria such as *Escherichia coli*, *Mannheimia succiniciproducens*, and *Basfia succiniproducens*. Most of these natural succinate producers utilizes the reductive branch of the TCA cycle for the production of succinate. While this reductive TCA pathway is the most carbon conserving pathway for succinate production, its transfer to other heterologous organisms is somewhat limited due to the membrane bound nature of fumarate reductase and specific redox partner. While some organisms have soluble fumarate reductases, these enzymes are typically significantly less active (17). To bypass this difficulty, some studies (18, 19) have used the glyoxylate shunt and achieves production of succinate under aerobic condition. Naturally, B. methanolicus MGA3 overproduces glutamate up to 55 g/L, indicating that its natural carbon flux to α-ketoglutarate is sufficiently high. Succinate is an intermediate of the TCA cycle and is produced from oxidative decarboxylation of α-ketoglutarate to succinyl-CoA followed by substrate

level phosphorylation of ADP to form succinate and ATP. However, overexpression of this pathway is potentially difficult due to the ordered multi-subunit nature of the α-ketoglutarate dehydrogenase. Here we propose to bypass α-ketoglutarate dehydrogenase by constructing a synthetic pathway leading to the synthesis of succinate through succinate semialdehyde. In this pathway, succinate is synthesized through decarboxylation of α-ketoglutarate to succinate semialdehyde by α-ketoglutarate decarboxylase (Kgd) and followed by an oxidation to succinate by succinate semialdehyde dehydrogenase (Ssd). This pathway was recently proposed as an alternative TCA cycle in cyanobacteria (20). The advantage of this pathway is that the two enzymes Kgd and Ssd are readily found in nature and are soluble proteins, which have been characterized previously (20, 21). B. methanolicus has an active respiration chain, which enables its rapid utilization of NADH for the regeneration NAD+ allows its growth on methanol using methanol dehydrogenase. Therefore, we expect that the consumption of NADH may serve as a driving force for our proposed pathway as it generates NAD(P)H during the production of succinate.

The goal of this research work is to evaluate the capability of methylotrophic bacteria B. methanolicus and M. extorquens for succinate production. in particular, we engineered B. methanolicus to express two independent pathways: Kgd-Ssd and glyoxylate pathway. however, the results showed minimal improvement in succinate production while having overexpressed and elevated enzyme activities, indicating either insufficient carbon flux entering the two pathway or intracellular succinate consumption is rapid. For M. extorquens, we optimized its growth condition and characterized nine promoters for gene expressions. We are currently in the progress of developing CRISPRi-based gene inhibition tools. Overall, while production of succinic acid from methanol is possible, more work is required for developing robust genetic tools and information regarding their natural metabolism.

貳、研究方法與過程

In the current work, we evaluated the the potential of converting methanol to succinate by using B. methanolicus and M. extorquens. For B. methanolicus, two distinct wild types are available: PB1 and MGA3. Here in this study, we initially

focused on B. methanolicus PB1 as it is commercially available from many deposit organizations. We then studied B. methanolicus MGA3 mutant, in which we refer to as MGC6 in this study. This mutant was isolated from a B. methanolicus MGA3 strain harboring a pNW33N plasmid that we received from a collaborating lab. B. methanolicus MGC6 lost the plasmid. M. extorquens AM1 was purchased from DSMZ collection.

Chemicals and reagents

All chemicals were purchased from Sigma-aldrich, J.T. Baker, or Amresco unless otherwise specified. High fidelity DNA polymerase used was KOD or KOD Xtreme purchased from Merck Biosciences. T4 polymerase was purchased from New England Biolabs. LB, Yeast extract, and tryptone were purchased from focusBio. Bacto agar was purchased from BD Bioscience.

Plasmid construction

All plasmids constructed in this study was done by either Gibson DNA assembly (22) or ligation independent assembly (23). A list of strains and plasmids used in this study is found in Table 2. *E. coli* strain XL1-blue was used for maintenance and propagation of all plasmids.

Cultivation of *B. methanolicus*

In this work, three different media were used to grow both *B. methanolicus* MGC6 and PB1:

SOB media: 20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.4 g/L $MgSO_4$, 0.186 g/L KCl with 200 mM methanol.

$MVcMY_{200}$ media: K2HPO4 3.8 g/L, NaH2PO4.H2O2.8 g/L, (NH4)2SO4 3.6 g/L, MgSO4.7H2O 0.25 g/L, FeSO4.7H2O 2 mg/L, CuSO4.5H2O 40 ,µg/L, H3BO3 30 µg/L, MnSO4.H2O 200 µg/L, ZnSO4.7H2O 200 µg/L, Na2MoO4.2H2O 47 µg/L, CaCl2.2H2O 5.3 mg/L, CoCl2.6H2O 40 µg/L, thiamine hydrochloride 50 µg/L, D-calcium pantothenate 50 µg/L, Riboflavin 50 µg/L, nicotinamide 50 µg/L, biotin 20 µg/L, folic acid 20 µg/L, vitamin B12 1 µg/L, L-methionine 224 mg/L, L-threonine 119 mg/L, Yeast extract 250 mg/L, Methanol 200 mM

M2YG: K2HPO4 3.8 g/L, NaH2PO4.H2O2.8 g/L, (NH4)2SO4 3.6 g/L,

MgSO4.7H2O 0.25 g/L, FeSO4.7H2O 2 mg/L, CuSO4.5H2O 40 µg/L, H3BO3 30 µg/L, MnSO4.H2O 200 µg/L, ZnSO4.7H2O 200 µg/L, Na2MoO4.2H2O 47 µg/L, CaCl2.2H2O 5.3 mg/L, CoCl2.6H2O 40 µg/L, thiamine hydrochloride 50 µg/L, D-calcium pantothenate 50 µg/L, Riboflavin 50 µg/L, nicotinamide 50 µg/L, biotin 20 µg/L, folic acid 20 µg/L, vitamin B12 1 µg/L, L-Glutamic acid monosodium 1.69 g/L, Yeast extract 2 g/L, Methanol 200 mM

Routine cultivation was done by cultivation on SOB agar solid medium for isolation of single colonies. Liquid cultivation was usually done by inoculating SOB medium with *B. methanolicus* colony or from frozen stock. Then liquid culture was incubated in 50 °C overnight. Subsequent day, the SOB culture was transferred to other mediums for growth or production test. Cell growth was measured using optical density of the culture at 600 nm. 200 µL of appropriately diluted cell culture was measured using Biotek Epoch 2 spectrophotometer.

Transformation of *B. methanolicus*

The same transformation protocol was used for *B. methanolicus* PB1 and MGC6. The transformation of *B. methanolicus* was conducted as described previously (24). SOB medium was inoculated with *B. methanolicus* cell stock from -80 °C and incubated in 50 °C overnight. Next day, preculture was used to inoculate a new culture. The new cultures were allowed for growth at 50 °C until OD600 reaches around 0.3. Then, the cells were harvested by centrifugation at 5000 x g for 20 minutes. The pellets were washed twice with EP buffer (1 mM HEPES, 25% PEG8000, pH7). Final resuspension was made to 100x concentration of the original cell density. Then 1 µg of DNA was added to 80-100 µL of cell. The mixture was allowed to incubate on ice for around 30 minutes. Then, the mixture was electroporated using Bio-Rad gene pulser II with settings of 200 Ω; 25 µF; 2.5 kV. The electroporation cuvette used was 0.2 cm gap aluminum cuvettes (BioRad). The electroporated cells were then transferred to 50 mL of SOB and allowed for 2-hour rescue. Subsequently, appropriate amount of chloramphenicol was added to the culture. The culture was incubated overnight. Next day, appropriate amount of culture was plated on SOB plate containing 5 µg/ml chloramphenicol for isolation of single colony transformants.

Product quantification

HPLC analysis. All organic acid metabolites were quantified using Agilent 1260 HPLC equipped with an Agilent HiPlex-H (700 x 7.7 mm) organic acid analysis column. A Bio-Rad Micro-Guard Cation H guard column (30 x 4.6 mm) was connected in front of the analysis column. The detectors used include diode array detector (DAD) and refractive index detector (RID). The column was maintained at 65 °C throughout the entire HPLC analysis method. The injection volume used was 20 µL. The mobile phase used was 5 mM H_2SO_4 at a constant flow rate of 0.6 mL/min. Concentration of succinate in the culture medium was calculated based on a standard curve made from HPLC analysis of standard succinate solutions with concentrations of 0.1 to 10 mM.

GC analysis. All alcohols and other volatile compounds were quantified by gas chromatography (GC) with barrier ionization detector (BID). The GC system used is a Shimadzu GC-2010 with an AoC-20S auto sampler and an AoC-20i Auto Injector. The used column was a DB-Wax capillary column (30 m length, 0.32 mm diameter, 0.50 µm film thickness). The GC oven temperature was initially held at 40°C for 3 min, then increased at a rate of 45 °C min-1 until 235 °C and held for 3 min. Injector temperature was held at 225 °C and FID detector was held at 330 °C. Injection volume was 1 µL, injected at a 15:1 split ratio. Helium was used as the carrier gas. 1-pentanol was used as internal standard.

Cultivation of *M. extorquens*

In this work, M. extorquens was cultivated in complex media nutrient broth, mineral media, and their combinations.

Nutrient broth: 5 g/L Peptone, 3 g/L meat extract

Mineral medium: 1% v/v methanol, 2 g/L (NH4)2SO4, 0.896 g/L MgSO4 · 7H2O, 1.304 g/L KH2PO4, 2.128 g/L Na2HPO4, 3.2 mg/L CaCl2 · 2H2O, 13.4 mg/L Na3C6H3O7 · 2H2O, 5 mg/L FeSO4 · 7H2O, 0.34 mg/L ZnSO4 · 7H2O, 0.18 mg/L MnCl2 · 4H2O, 0.24 mg /L CuSO4 · 5H2O, 0.46 mg/L CoCl2 · 6H2O, and 0.028 mg/L H3BO3.

Mixed medium: 50% nutrient broth and 50% mineral medium v/v.

Transformation of M. extorquens

Cells were grown in “Mix medium”(containing 1% v/v methanol) until the culture reached an OD_{600}=0.6~0.8. Cells were harvested by centrifugation(1800 X g, 10 min, 4℃) and washed twice with ice-cold sterile 10% (v/v) glycerol solution. The cell suspension was concentrated 10-fold in 10% glycerol, dispensed in 400-ul aliquots and kept at -80℃. Electro-competentcells (100 ul) were mixed with DNA solution (500 ng) in a 0.2-cm gap cuvette chilled on ice. Electroporation was carried out using a Gene Pulser (Bio-Rad) with the follow-ing parameters: 2.5 kV, 400Ω,25 uF, to a final field strength of 12.5 kV cm^{-1}. After cells had been pulsed,1 ml of ice-cold sterile “Mix medium” was added to the cuvette immediately, the cell suspension transferred into a test tube, and then incubated at 30℃ for 24 h. Transformed clones were selected “Mix medium” agar with appropriate antibiotics (kanamycin : 50 ug/ml).

Isocitrate lyase assay

To assay isocitrate lyase, an overnight culture was inoculated into fresh SOB media at 1% v/v. These cultures were allowed to grow for 8 days in which the culture was used to test succinate production. Subsequent to quantification of products, the cultures were then centrifuged at 5000 x rpm for 20 minutes into pellet. The pellets were resuspended with lysis buffer (25 μmol sodium potassium phosphate buffer (pH 6.8), 5 mM $MgCl_2$, 1 mM EDTA) using 5% of the original culture volume. Then, these concentrated cultures were homogenized with minibead beater using 0.1 mm beads. The mixtures were then centrifuged at 14,000 rpm 10 minutes. The supernatant was collected as soluble cell lysate and used to measure isocitrate lyase activity. The isocitrate lyase reaction was prepared by adding isocitrate 50 mM, 4 mM 16 mM phenylhydrazine HCl, crude extract 50 μL, with reaction buffer up to total 200 μL. The glyoxylate formed in the reaction spontaneously reacts with phenylhydrazine to form phenylhydrazone, which absorbs light at 324 nm that we monitored using Bioteck epoch II microplate reader. The amount of glyoxylate formed was calculated based on a standard curve constructed with glyoxylate standard solution of concentration range 0.0625 to 50 mM.

Promoter expression assay

To assay the promoter strength of 9 promoters (PmxaF、Ptuf、PfumC、PcoxB、PBAD、Plac、PT5、Ptrc, and PL_{lacO1}), we fused red fluorescent protein gene behind each promoter on individual plasmids. RFP can be measured via excitation wavelength of 585 nm and emission of 610 nm. The transformed M. extorquens strains harboring each of the rfp expressing plasmids were inoculated in 4 mL mix medium and allowed to grow for 48 hours. Appropriate inducers (1 mM IPTG or arabinose) were added to the cultures 24 hours post inoculation. Subsequently, 200 μL was taken out for fluorescence and optical density measurement. To normalize the fluorescence, we took the fluorescence measured subtract the background fluorescence obtained from the negative control strain harboring only pCM66T and then divided by the optical density (OD600) of the cells.

參、主要發現與結論

Overexpression of glyoxylate shunt in B. methanolicus

The glyoxylate pathway is frequently used for aerobic production of succinate in *E. coli*. We identified that B. methanolicus codes for isocitrate lyase (aceB) and malate synthase (aceA), the two genes that completes glyoxylate pathway. However, under normal metabolism, aceBA may not be active. Therefore, we overexpressed aceBA. Here, for this experiment, we chose to use both B. methanolicus MGC6 and PB1. However, as PB1 transformation was unsuccessful, potentially due to excessive metabolic burden.

The plasmids for overexpressing the glyoxylate pathway were constructed by DNA assembly of a vector fragment with the genes required to complete the glyoxylate pathway. However, here Pmdh was chosen for its relative higher expression. Also, as reported in many previous work, the flux to oxaloacetate is important for succinate production using glyoxylate pathway, therefore, we cloned pck and pyc individually

with the genes of glyoxylate pathway. The plasmids are depicted below in figure 2. pAH8 expresses only aceBA. pAH9 expresses aceBA with pckA. pAH13 expresses aceBA with pyc. All genes chosen are native to B. methanolicus

Next, we transformed the above plasmids into both B. methanolicus PB1 and MGC6. While MGC6 transformation was successful and verified by colony PCR, PB1 transformation failed, potentially indicating metabolic burden for PB1. While we will continue to try PB1 transformation with these glyoxylate pathway overexpression plasmids, we continued the characterization with transformed MGA3. Interestingly, wildtype MGC6 yielded no observable succinate, which is different from PB1. Growth (Figure 3) between all strains tested were similar, indicating no significant perturbation in metabolism by the inclusion of these plasmids. Unfortunately, MGC6 expressing the glyoxylate pathway plasmids showed no difference when compared to MGC6 expressing empty plasmid (Figure 4). To further analyze what was going on with the overexpressed glyoxylate pathway genes, we conducted in vitro crude extract enzyme assay to assess if aceBA were properly overexpressed. Here we tested aceB as a representation for the pathway. As shown in Figure 5, isocitrate lyase activity was higher in the strains overexpressing aceB gene, indicating successful overexpression. These results indicate that the activity of glyoxylate pathway remains insufficient to support succinate production. This is likely due to the overly active glutamate synthesis in B. methanolicus MGC6 or an active TCA cycle.

Since SOB is complex media, it is likely that other compounds in SOB shadowed succinate, rendering it undetectable. Therefore, we evaluated a more defined media MVcMY200. This media is based on minimum salt media with addition of vitamin solutions and small amounts of yeast extract. Using this media to cultivate MGC6 harboring either pHP13 or pAH9, we observed succinate in the culture samples (Figure 6). There was no significant difference in growth of between the two strains. Both strains secreted succinate into the solution up to 60 mg/L at day two. However, gradually the succinate was re-uptaken by the culture as the level of succinate decreased. This result is consistent with above experiment that while isocitrate lyase was expressed, succinate production did not increase. We expect that this may due to excessive carbon entering glutamate biosynthesis. To check for glutamate secretion, we

measured glutamate in the culture. However, contrary to our expectation, the amount of glutamate detected in MGC6 cultures was similar to that in the fresh medium, indicating that glutamate was not produced (Figure 7). Therefore, it is likely that the current limitation in engineering succinate production from methanol using B. methanolicus is the oxidation of succinate by succinate dehydrogenase or insufficient flux entering TCA cycle. To test the second possibility, next we overexpress genes responsible for enhancing TCA cycle flux.

Overexpression of TCA cycle genes in B. methanolicus

Previously, we showed that supplementing α-ketoglutarate into the culture medium of Bacillus methanolicus PB1 increased its succinate secretion significantly. Here, we selected native ppc, gltA, and acn genes for overexpression (Figure 8). These gene overexpressions are applicable to both enhancing TCA cycle flux and subsequently for building glyoxylate pathway. Using the broad-range-host vector pHP13 for Gram-positive bacteria as the backbone and Pfba, promoter of native fructose-bisphosphate aldolase, as the promoter, we constructed three different plasmids. Plasmid pJT-S expresses only gltA, pJT-T expresses gltA and ppc, and pJT-U expresses gltA, ppc, and acn (Figure 2). The gene fragments for ppc, gltA, and acn were individually amplified by PCR using primers specific to the genes with B. methanolicus genomic DNA as template. These fragments were then assembled using Gibson assembly method with a pHP13::Pfba fragment amplified from pJT-J, a plasmid that we previously constructed. The plasmids were then check with colony PCR and sequenced.

Next, we transformed the above constructed plasmids into B. methanolicus PB1. Here, we chose to use PB1 first because it is commercially available and has previously shown decent amounts (100 mg/L) of succinate from its cultivation on methanol media containing α-ketoglutarate. Transformation of B. methanolicus PB1 remains low in efficiency compared to commonly used bacteria such as E. coli. Nonetheless, successful transformants were isolated. These strains were subsequently cultivated to see if succinate secretion increased. Growth of the different strains were comparable (Figure 3), indicating little or no metabolic stress. However, contrary to our expectation,

strains expressing additional genes from TCA cycle did not improve succinate production when compared to negative control which harbors an empty plasmid (Figure 3). Upon analysis, we noticed that the ppc cloned is not ideal for the conversion of PEP to oxaloacetate. Native B. methanolicus uses PEP carboxykinase (Pck) and pyruvate carboxylase (pyc) instead of Ppc. The ppc gene that we cloned codes for phosphopantothenoylcysteine decarboxylase instead of PEP carboxylase. Therefore, we constructed three new plasmids pTC11, pTC12, and pTC13, expressing ppc from Thermosynechococcus elongatus (Figure 9).

Next, we attempted to transform both PB1 and MGC6 with pTC11, pTC12, and pTC13. However, transformation of PB1 failed. Therefore we proceeded this experiment with MGC6. However, as shown in figure 11, there is no significant increase in succinate secretion between MGC6 harboring empty pHP13 and the plasmids expressing TCA cycle genes. Whereas the growth remained similar. We analyzed the glutamate production. As shown in figure 7, no succinate was produced by strains overexpressing TCA cycle genes. Therefore, we believe that the current bottleneck is in the rapid degradation of succinate via succinate dehydrogenase. Therefore, to construct a better strain of succinate producing B. methanolicus, knock out tools are necessary.

As a potentially fast way to test if succinate dehydrogenase inhibition would increase succinate secretion, we tried to inhibit succinate dehydrogenase via malonate feeding. Malonate is a three carbon diacid with structural similarity to succinate and has been shown to inhibit succinate dehydrogenases. As the results shown in figure 12, growth of B. methanolicus was slightly inhibited after 2 days of cultivation by addition of 10 mM malonate. No significant difference was observed in the succinate secretion.

Increased succinate production via media adjustment

Previously we showed that supplementing α-ketoglutarate into the culture increased succinate production by B. methanolicus. Therefore, we sought to see the effect of supplementing the media with other TCA cycle intermediates. Due to the high cost of isocitrate, it was not included in this test. In addition, we compared the effect of

changing carbon source from methanol to mannitol for B. methanolicus. As the results shown in figure 13, methanol was the better carbon source for B. methanolicus growth, despite that mannitol was a sugar carbon source recommended. More importantly, succinate production increased with glutamate addition, with the highest production titer reaching 160 mg/L. It is interesting that citrate addition significantly inhibited cell growth. These results indicate that glutamate in the media is likely to be quickly uptaken by B. methanolicus.

Next, we evaluated the effect of glutamate feeding using MGC6 strain. As shown in figure 14, growth of MGC6 was rapid, reaching stationary phase within the first day. Succinate secretion increased through the 10 day period tested, somewhat consistent with that observed from PB1. Since addition of glutamate helped succinate production, we then attempted co-feeding glutamate with malonate, hoping to see a enhanced difference. However, as shown in figure 15, no significant difference was observed with and without malonate feeding, therefore indicating that either malonate does not inhibit B. methanolicus succinate dehydrogenase or that there are alternative pathways which could drain succinate flux.

Growth of M. extorquens in complex media

Methylobacterium extorquens AM1 is a mesophilic methylotroph that has been engineered to produce different bioproducts from methanol. Initially it was not selected for succinate production because it consumes succinate as a natural organic substrate. However, as B. methanolicus is difficult to manipulate, this year we propose to study M. extorquens AM1 to evaluate its potential for succinate production. To do that, we analyzed its growth rate, general manipulations, and in the progress of developing CRISPRi-based inhibition tools to prevent succinate consumption.

We first attempt to understand and characterize the growth behavior of M. extorquens AM 1 in complex media. As recommended by collection center DSMZ, we cultivated M. extorquens AM 1 in Nutrient broth with either 0.5 or 1 % v/v methanol. We also tested the amount of inoculation with either a loop of cells or a single colony.

In addition, we also tested growth in flask vs. test tube. As results shown in Figure 16, growth was best observed when culture was grown in flasks with methanol concentration lower than 1%. However, due to its natural ability to synthesize cellulose (25), M. extorquens AM1 aggregates in solution and makes optical density measurements difficult. Due to aggregation, maximum optical density measured was around 0.4. Here it seems that the growth rate of M. extorquens AM1 is slower than B. methanolicus. However, due to its mesophilic property, we will consider it as a potential host for succinate production.

Optimized M. exotrquens growth

In order for us to develop engineering methods and recombinant AM1 strains, we need to first be able to cultivate AM1 without aggregation. Thinking that nutrients lead to rapid growth and synthesis of cellulose as energy storage compounds, we tested its growth under mineral minimal media (26). As shown in figure 17, growth of AM1 is significantly better than in nutrient broth. Interestingly, growth rates were not significantly reduced. Furthermore, cellular aggregation was significantly reduced as well. However, considering that it takes 2 days to reach OD 0.6 which is much slower than ideal. We attempted to grow AM1 under mix medium, which is made by 50% mineral medium and 50% nutrient broth. AM1 growth was significantly improved in mixed medium (Figure 18). Therefore, mixed medium will be used for downstream experiments.

Next, we conducted a detailed analysis of the growth curve of AM1 in mix medium with different amounts of initial methanol added. This is important as excessive methanol is toxic even to methylotrophs due to the production of formaldehyde. As shown in figure 19, 0.1 % methanol was insufficient to promote continuous growth and correspondingly culture stopped growing at OD 0.5. Using 0.5 % methanol achieved fast growth rate, however cell growth stopped at OD 2. 1% and 2% methanol yielded highest stationary cell density. However, 2% caused a longer lag phase, potentially due to formation of toxic formaldehyde. 3% of methanol resulted in an even longer lag phase. Therefore 0.5 - 1% methanol seems best condition to work with.

Assessment of promoter strengths in M. extorquens

Succinate is often used as the non-methanol organic substrate for M. extorquens AM1 growth. This is because succinate is metabolized through the ethylmalonyl-CoA pathway. As its natural metabolism on methanol, M. extorquens AM1 also uses ethylmalonyl-CoA pathway. Within the ethylmalonyl-CoA pathway, succinate dehydrogenase converts succinate to fumarate. Looking at the association of serine cycle and ethylmalonyl-CoA pathway, we think that we may be able to inhibit succinate dehydrogenase despite its essential role in ethylmalonyl-CoA pathway. If not complete inhibition, we should be able to produce if partial inhibition is possible. For that purpose, we will attempt this task using CRISPRi.

To develop CRISPRi tools, we first assessed different promoters for their strengths in AM1. We selected 9 promoters: PmxaF、Ptuf、PfumC、PcoxB、PBAD、Plac、PT5、PtrcOI, and PacOI. In particular, PmxaF、Ptuf、PfumC、PcoxB have been documented before in the literature (27). To measure the promoter strength, we cloned red fluorescent protein (rfp) gene behind each promoter and inserted this synthetic operon into a broad host cloning vector pCM66T available on addgene. The resulting plasmids were then introduced into AM1 via electroporation. The transformed cells were then assayed for their fluorescence. As shown in figure 20, plasmid pYA21, containing PTRCO1, induced with 1 mM IPTG yielded highest normalized fluorescence out of all of the promoters tested. Interestingly, PmxaF、Ptuf、PfumC、PcoxB showed minimal fluorescence. We hypothesize that this may due to removal of their native 5' untranslated regions which was necessary for use of these plasmids to drive sgRNA expression for CRISPRi.

Next, we constructed plasmids that aim to knock down succinate dehydrogenase genes (sdhA, sdhB, sdhC, and sdhD). However, the construction of these plasmids were unsuccessful and we are currently developing solutions by sequentially clone each feature one at a time into the plasmid.

肆、參考文獻

1. Beauprez JJ, De Mey M, & Soetaert WK (2010) Microbial succinic acid production: Natural versus metabolic engineered producers. *Process Biochem* 45(7):1103-1114.
2. Lin H, Bennett GN, & San KY (2005) Genetic reconstruction of the aerobic central metabolism in Escherichia coli for the absolute aerobic production of succinate. *Biotechnol Bioeng* 89(2):148-156.
3. Tan Z, Zhu X, Chen J, Li Q, & Zhang X (2013) Activating phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in combination for improvement of succinate production. *Appl Environ Microbiol* 79(16):4838-4844.
4. Lee SJ*, et al.* (2005) Metabolic engineering of Escherichia coli for enhanced production of succinic acid, based on genome comparison and in silico gene knockout simulation. *Appl Environ Microbiol* 71(12):7880-7887.
5. Stols L & Donnelly MI (1997) Production of succinic acid through overexpression of NAD(+)-dependent malic enzyme in an Escherichia coli mutant. *Applied and Environmental Microbiology* 63(7):2695-2701.
6. Lee SJ, Song H, & Lee SY (2006) Genome-based metabolic engineering of Mannheimia succiniciproducens for succinic acid production. *Applied and Environmental Microbiology* 72(3):1939-1948.
7. Zhang XL*, et al.* (2009) Metabolic evolution of energy-conserving pathways for succinate production in Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America* 106(48):20180-20185.
8. Hanson RS*, et al.* (1996) Production of L-lysine and some other amino acids by mutants of B-methanolicus. *Microbial Growth on C(1) Compounds*:227-236.
9. Brautaset T*, et al.* (2003) Role of the Bacillus methanolicus citrate synthase II gene, citY, in regulating the secretion of glutamate in L-lysine-secreting mutants. *Applied and Environmental Microbiology* 69(7):3986-3995.
10. Sirirote P, Yamane T, & Shimizu S (1988) L-Serine Production from Methanol and Glycine with an Immobilized Methylotroph. *Journal of Fermentation Technology* 66(3):291-297.
11. Motoyama H, Anazawa H, Katsumata R, Araki K, & Teshiba S (1993) Amino-Acid Production from Methanol by Methylobacillus-Glycogenes Mutants - Isolation of L-Glutamic Acid Hyper-Producing Mutants from M-Glycogenes Strains, and Derivation of L-Threonine and L-Lysine-Producing Mutants from Them. *Biosci Biotech Bioch* 57(1):82-87.
12. Hu B & Lidstrom ME (2014) Metabolic engineering of Methylobacterium extorquens AM1 for 1-butanol production. *Biotechnology for biofuels* 7.

13. Naerdal I, Pfeifenschneider J, Brautaset T, & Wendisch VF (2015) Methanol-based cadaverine production by genetically engineered Bacillus methanolicus strains. *Microb Biotechnol* 8(2):342-350.
14. Sonntag F*, et al.* (2015) High-level production of ethylmalonyl-CoA pathway-derived dicarboxylic acids by Methylobacterium extorquens under cobalt-deficient conditions and by polyhydroxybutyrate negative strains. *Applied microbiology and biotechnology* 99(8):3407-3419.
15. Sonntag F*, et al.* (2015) Engineering Methylobacterium extorquens for de novo synthesis of the sesquiterpenoid alpha-humulene from methanol. *Metabolic engineering* 32:82-94.
16. Brautaset T, Jakobsen OM, Flickinger MC, Valla S, & Ellingsen TE (2004) Plasmid-dependent methylotrophy in thermotolerant Bacillus methanolicus. *Journal of bacteriology* 186(5):1229-1238.
17. Coustou V*, et al.* (2005) A mitochondrial NADH-dependent fumarate reductase involved in the production of succinate excreted by procyclic Trypanosoma brucei. *Journal of Biological Chemistry* 280(17):16559-16570.
18. Okino S*, et al.* (2008) An efficient succinic acid production process in a metabolically engineered Corynebacterium glutamicum strain. *Applied microbiology and biotechnology* 81(3):459-464.
19. Lin H, Bennett GN, & San KY (2005) Metabolic engineering of aerobic succinate production systems in Escherichia coli to improve process productivity and achieve the maximum theoretical succinate yield. *Metabolic engineering* 7(2):116-127.
20. Zhang S & Bryant DA (2011) The tricarboxylic acid cycle in cyanobacteria. *Science* 334(6062):1551-1553.
21. Jaeger M, Rothacker B, & Ilg T (2008) Saturation transfer difference NMR studies on substrates, and inhibitors of succinic semialdehyde dehydrogenases. *Biochem Bioph Res Co* 372(3):400-406.
22. Gibson DG*, et al.* (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* 6(5):343-345.
23. Machado HB, Dekishima Y, Luo H, Lan EI, & Liao JC (2012) A selection platform for carbon chain elongation using the CoA-dependent pathway to produce linear higher alcohols. *Metabolic engineering* 14(5):504-511.
24. Jakobsen OM*, et al.* (2006) Upregulated transcription of plasmid and chromosomal ribulose monophosphate pathway genes is critical for methanol assimilation rate and methanol tolerance in the methylotrophic bacterium Bacillus methanolicus. *Journal of bacteriology* 188(8):3063-3072.
25. Delaney NF*, et al.* (2013) Development of an Optimized Medium, Strain and

High-Throughput Culturing Methods for Methylobacterium extorquens. *Plos One* 8(4).

26. Cui LY, *et al.* (2017) Medium redesign for stable cultivation and high production of mevalonate by recombinant Methtylobacterium extorquens AM1 with mevalonate synthetic pathway. *Biochem Eng J* 119:67-73.
27. von Borzyskowski LS, Remus-Emsermann M, Weishaupt R, Vorholt JA, & Erb TJ (2015) A Set of Versatile Brick Vectors and Promoters for the Assembly, Expression, and Integration of Synthetic Operons in Methylobacterium extorquens AM1 and Other Alphaproteobacteria. *Acs Synth Biol* 4(4):430-443.

伍、圖表

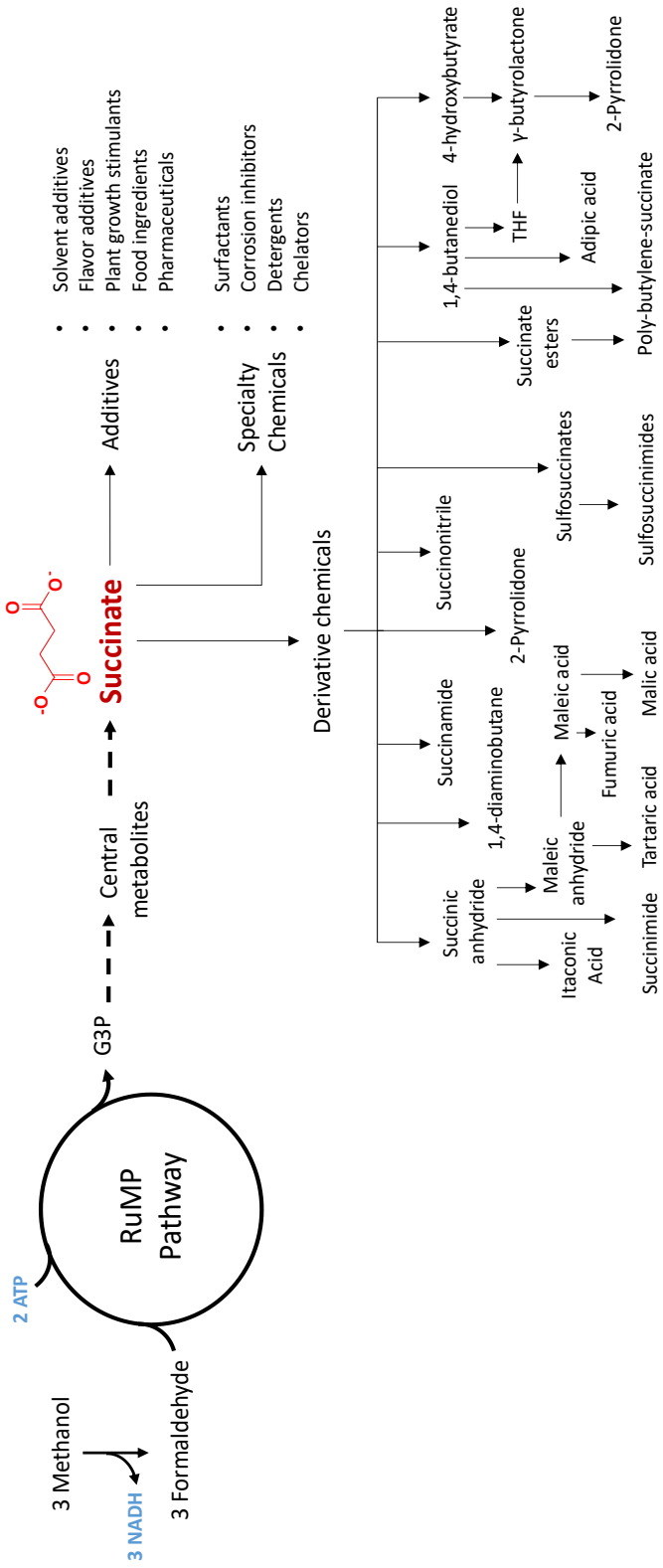


Figure 1. Schematics for methanol-based succinate production and applications of succinate

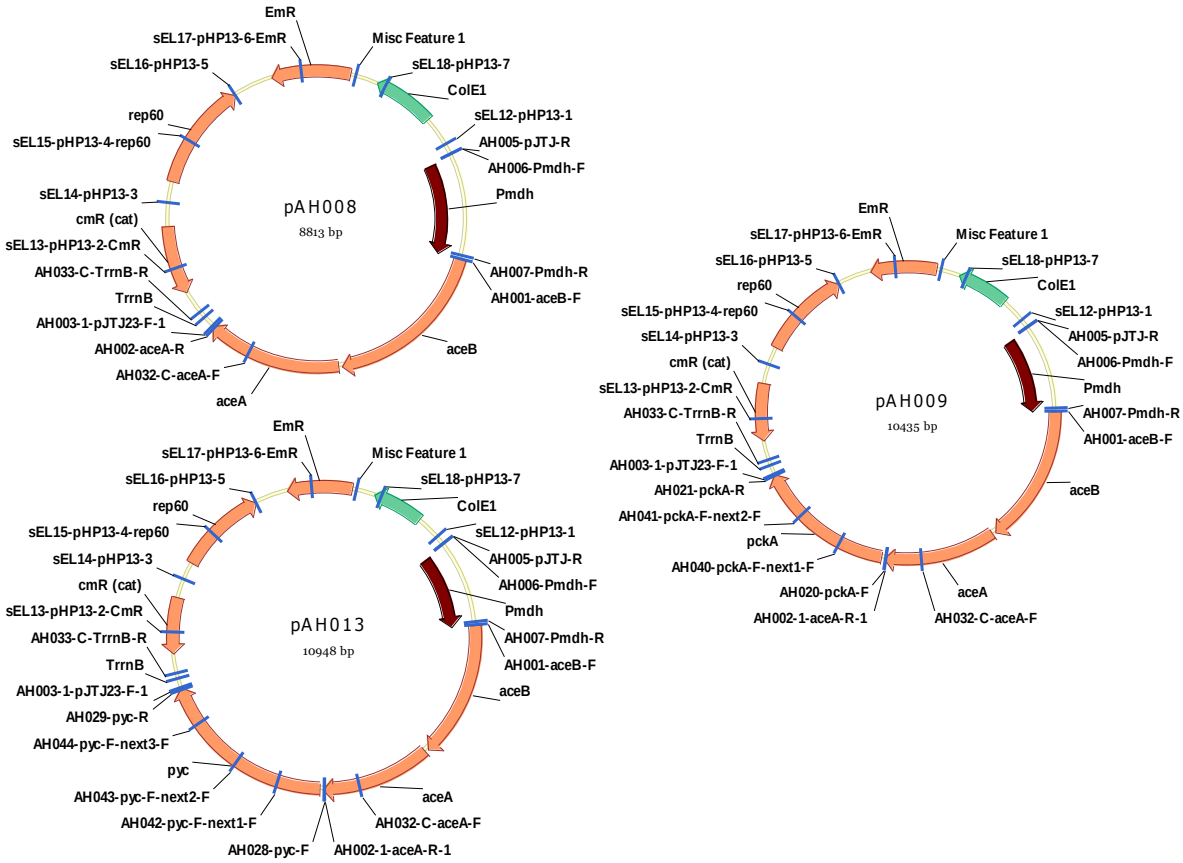


Figure 2. Plasmid maps for expressing native B. methanolicus glyoxylate pathway genes and gene responsible for oxaloacetate biosynthesis.

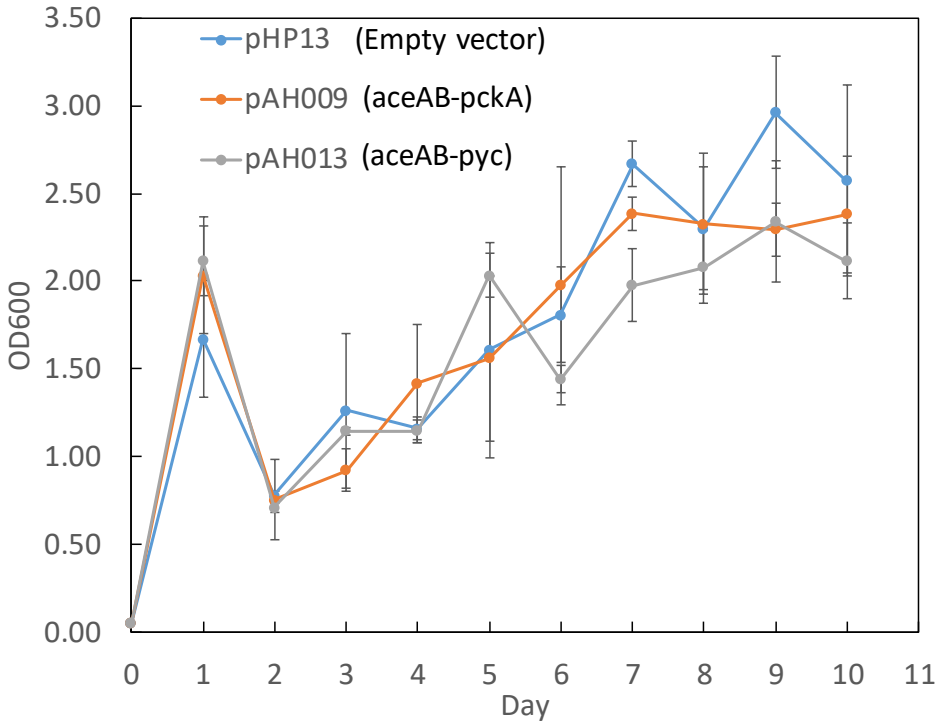


Figure 3. Growth of B. methanolicus MGA3 transformed with plasmids containing the glyoxylate pathway and gene responsible for oxaloacetate biosynthesis. SOB(methanol)

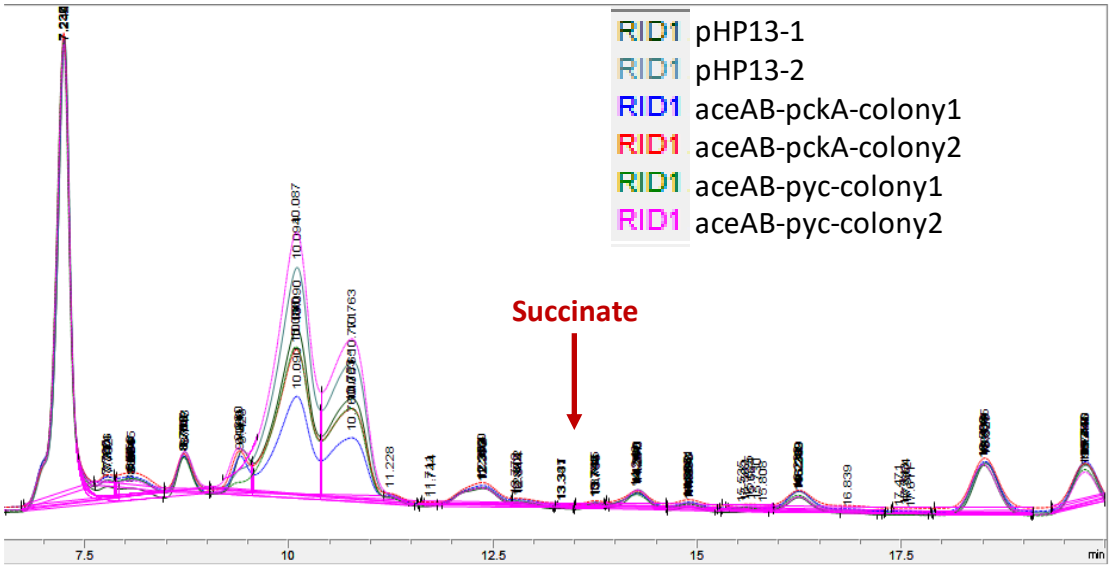


Figure 4. HPLC chromatogram of B. methanolicus MGC6 harboring either pHP13, pAH9, or pAH13 culture medium after 10 days. SOB was used for this cultivation. No clear succinate peak was observed.

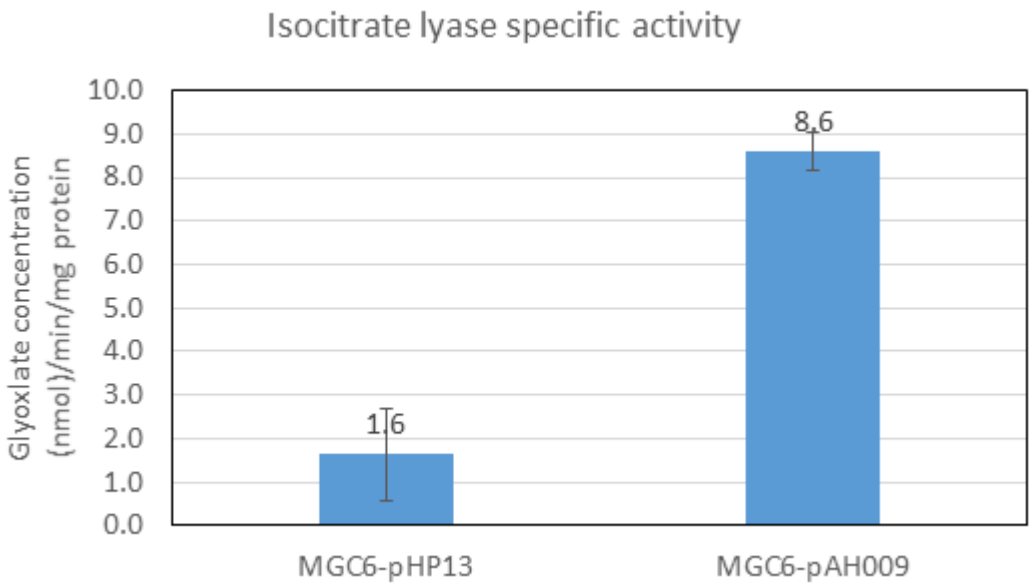


Figure 5. Comparison of isocitrate lyase activity between negative control (wild type MGC6 strain with empty pHP13 vector) and aceBA overexpression strain (MGC6 harboring pAH009).

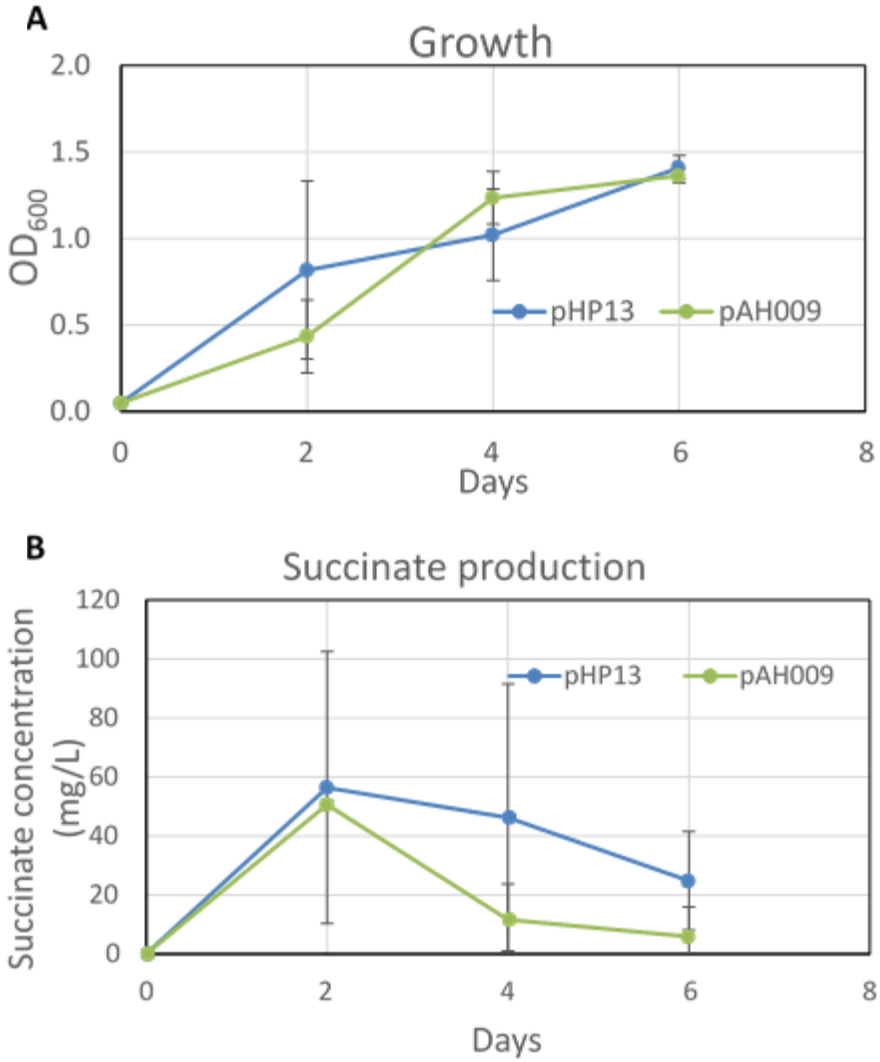


Figure 6. (A) Growth and (B) production of succinate by wildtype MGC6 and MGC6 harboring glyoxylate pathway in $MVcMY_{200}$ medium.

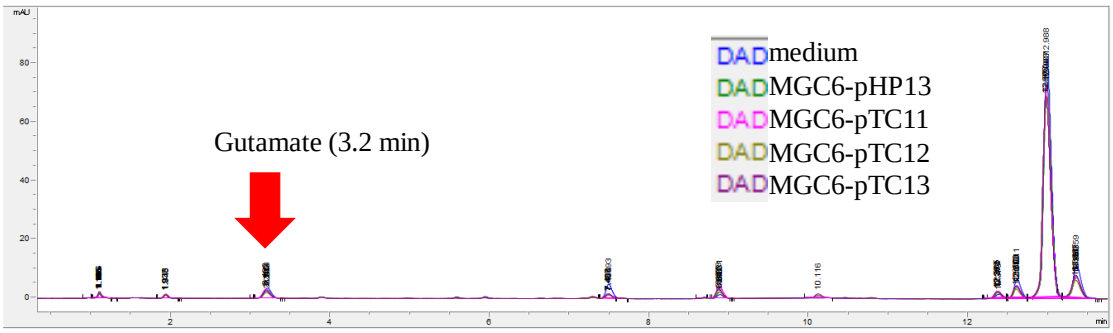


Figure 7. HPLC Chromatogram showing the presence of glutamate in MVcMY200 medium and culture broth of MGC6 cultures expressing either empty plasmid pHP13 or plasmids expressing TCA cycle genes.

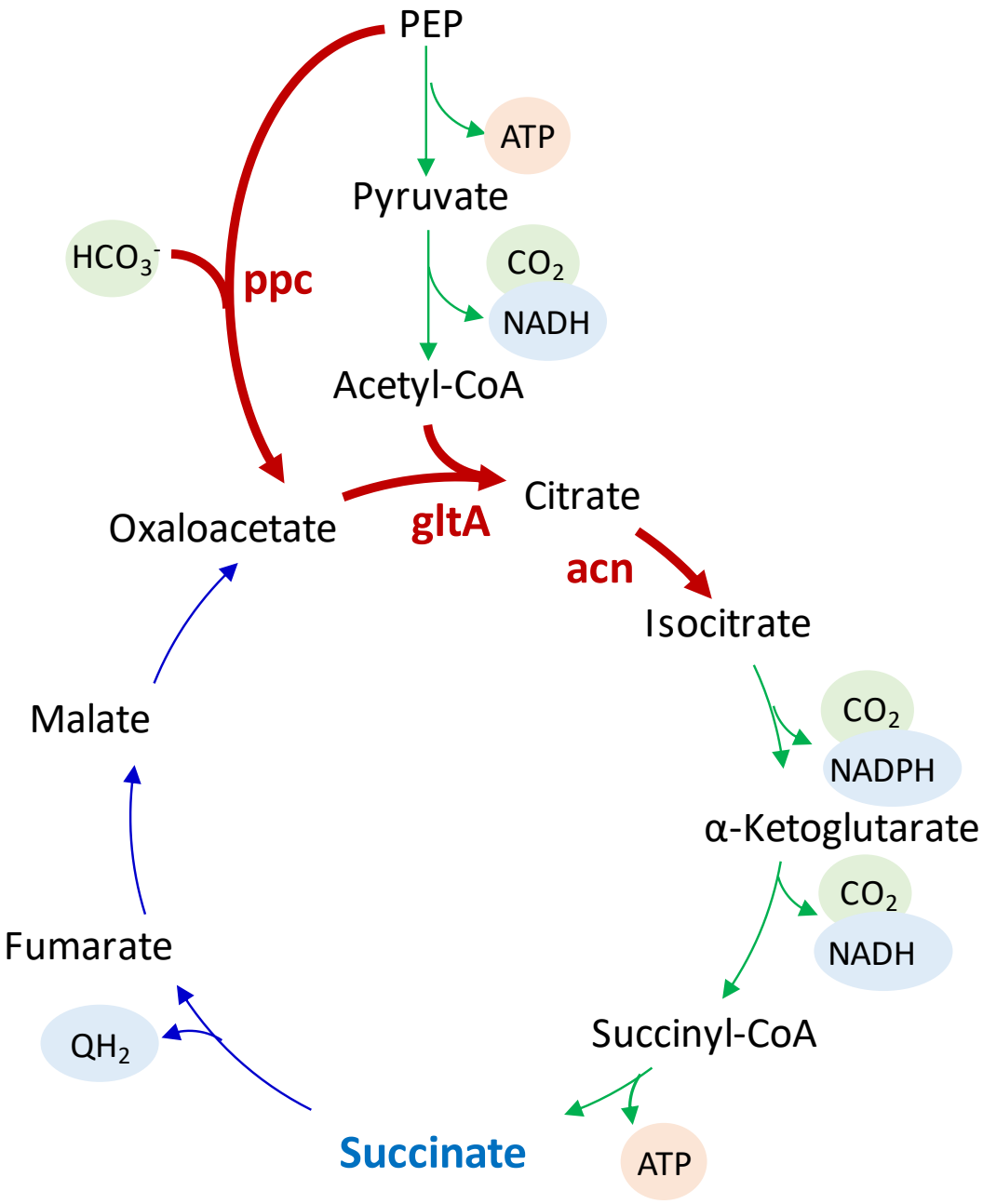


Figure 8. Oxidative TCA cycle for succinate synthesis. Genes highlighted represent those overexpressed.

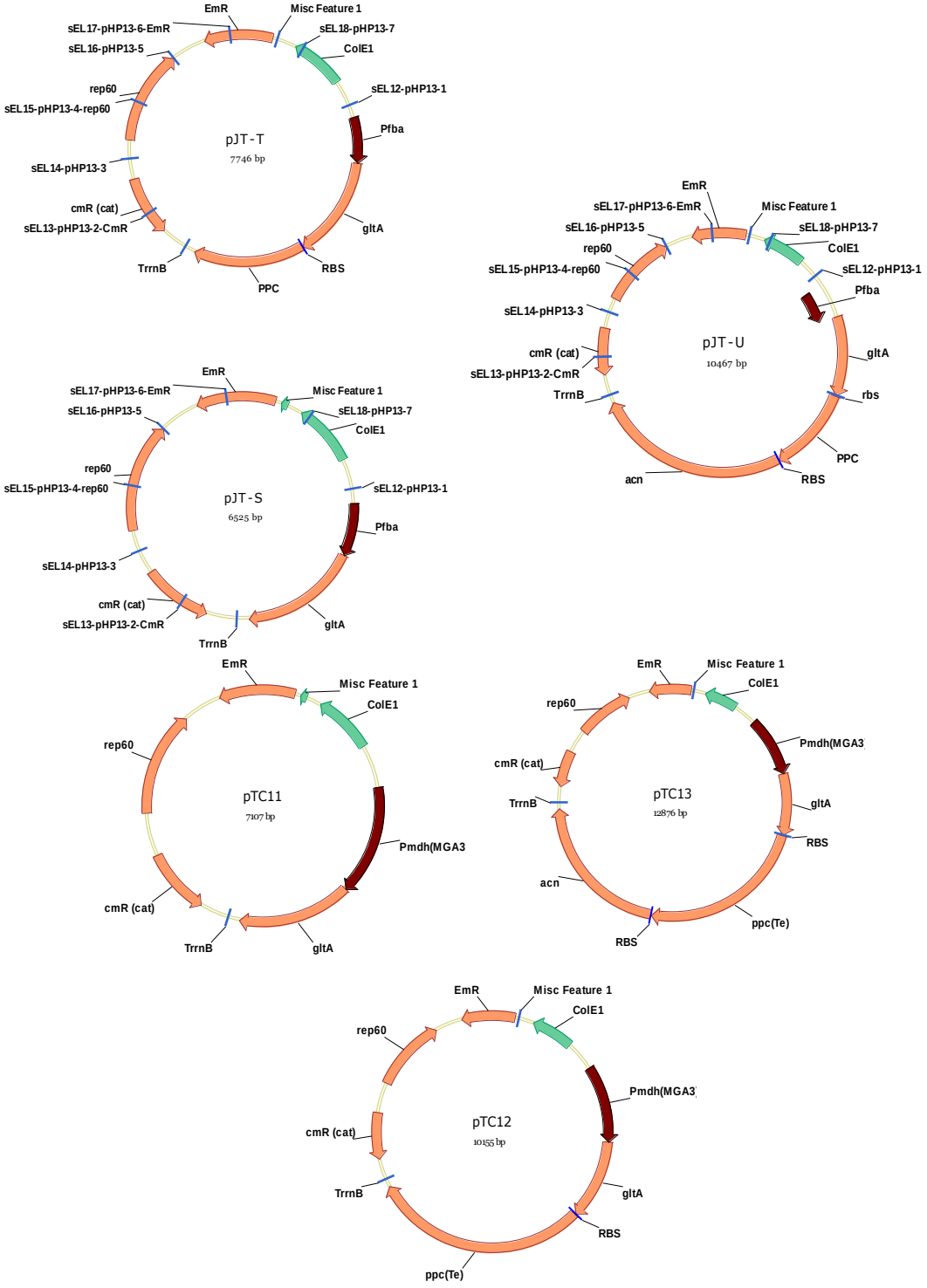


Figure 9. Plasmid maps for expressing native B. methanolicus ppc, gltA, and can using different promoter. Here pJT-T, and pJT-U used a non-specific ppc gene, whereas in pTC12 and pTC13, ppc used was a specific PEP carboxylase from T. synechococcus.

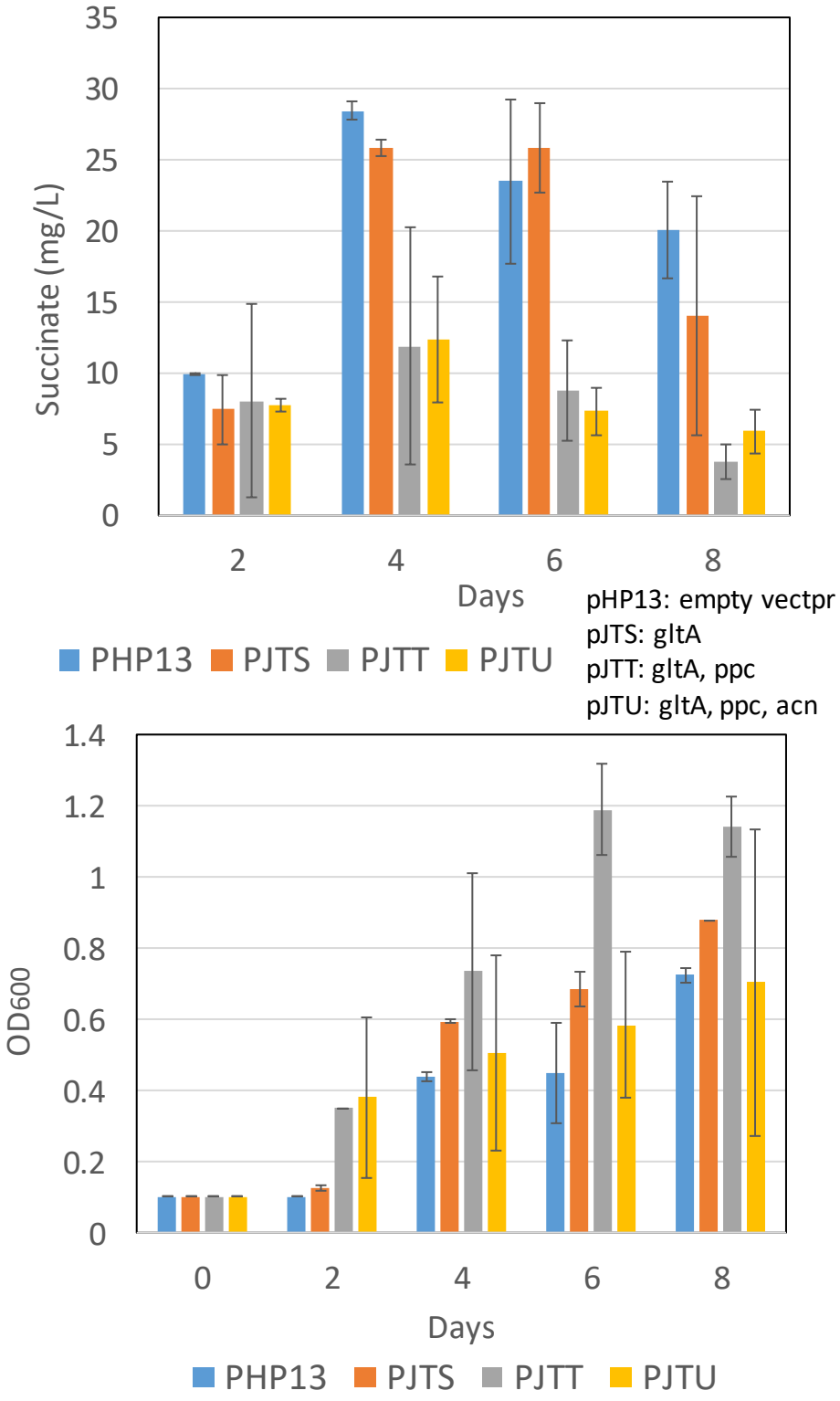


Figure 10. Growth and succinate secretion by strains transformed with plasmids that allow overexpression of TCA cycle genes.

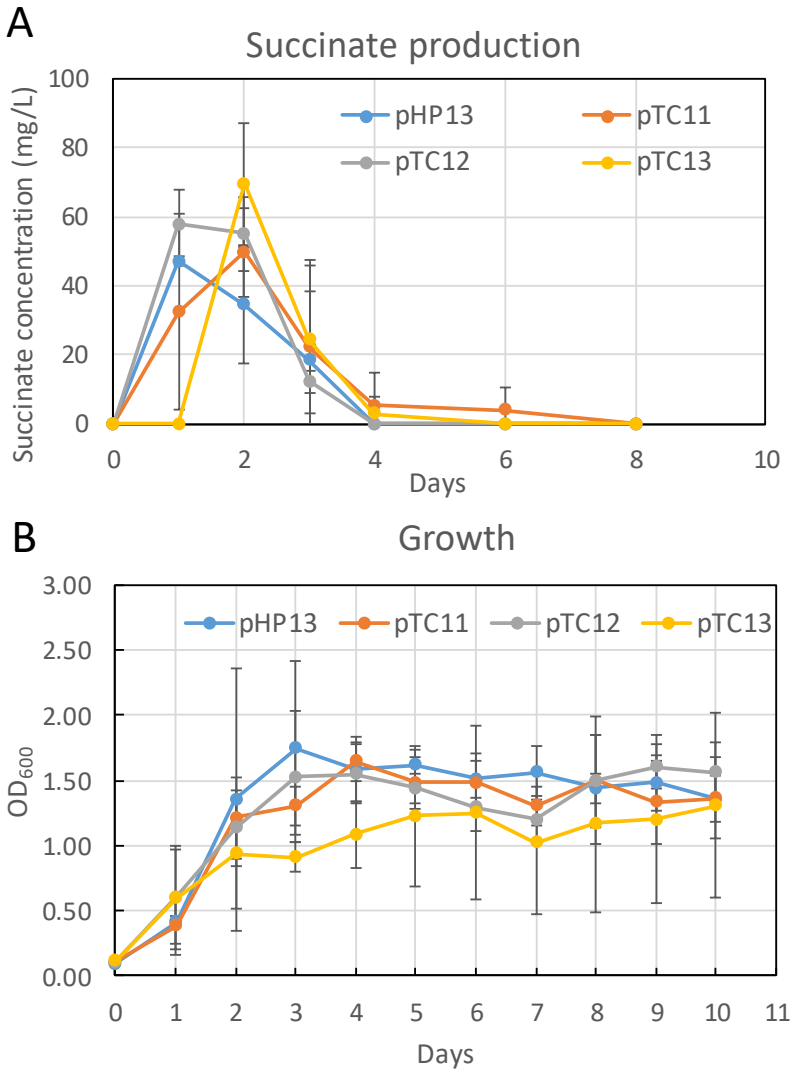


Figure 11. (A) Succinate production and (B) growth of strain MGC6 harboring different plasmids for expressing TCA cycle genes.

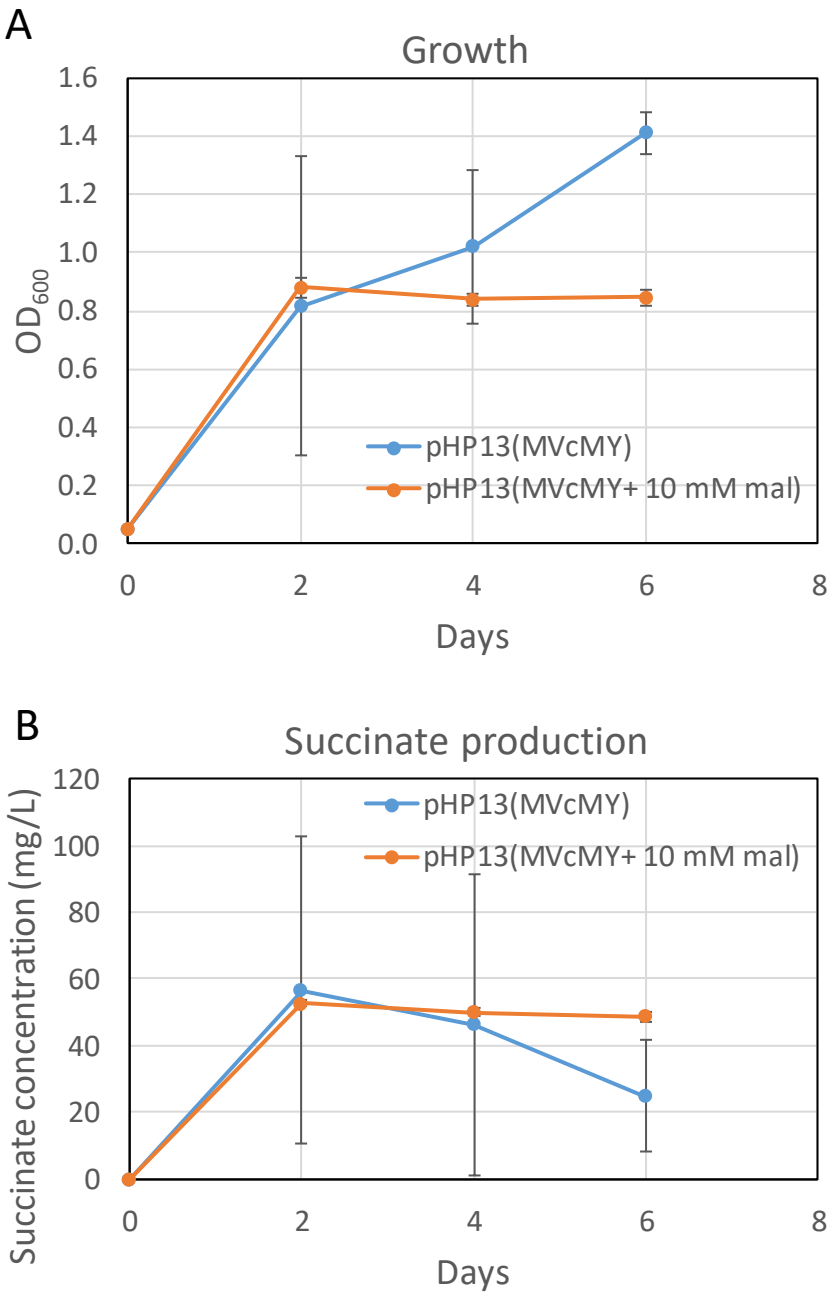


Figure 12. (A) growth and (B) production of succinate by MGC6 harboring empty plasmid pHP13 cultivated in $MVcMY_{200}$ with or without malonate.

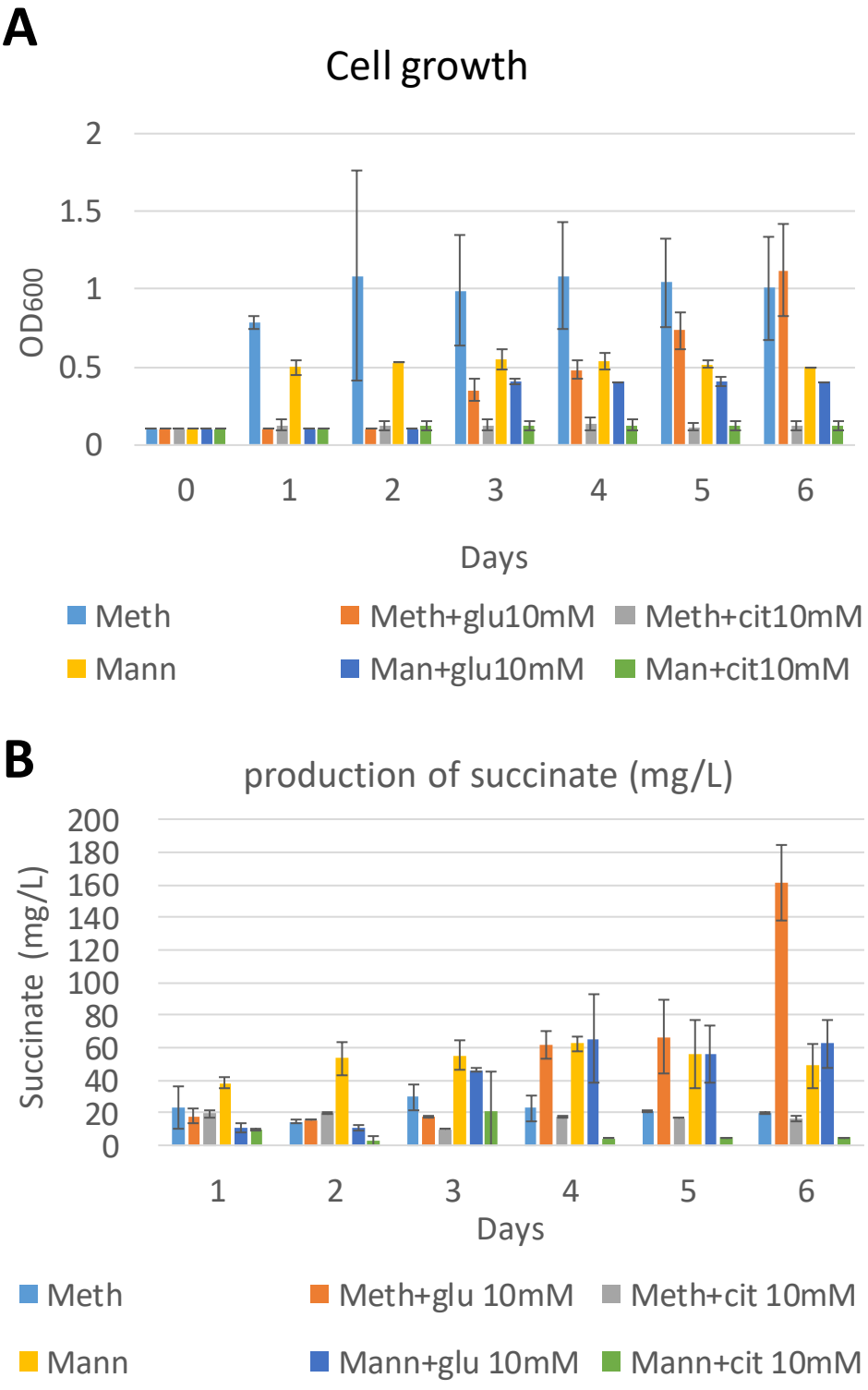


Figure 13. (A) Growth and (B) production of succinate by PB1 with either methanol or mannitol as main carbon source and citrate or glutamate as supplement.

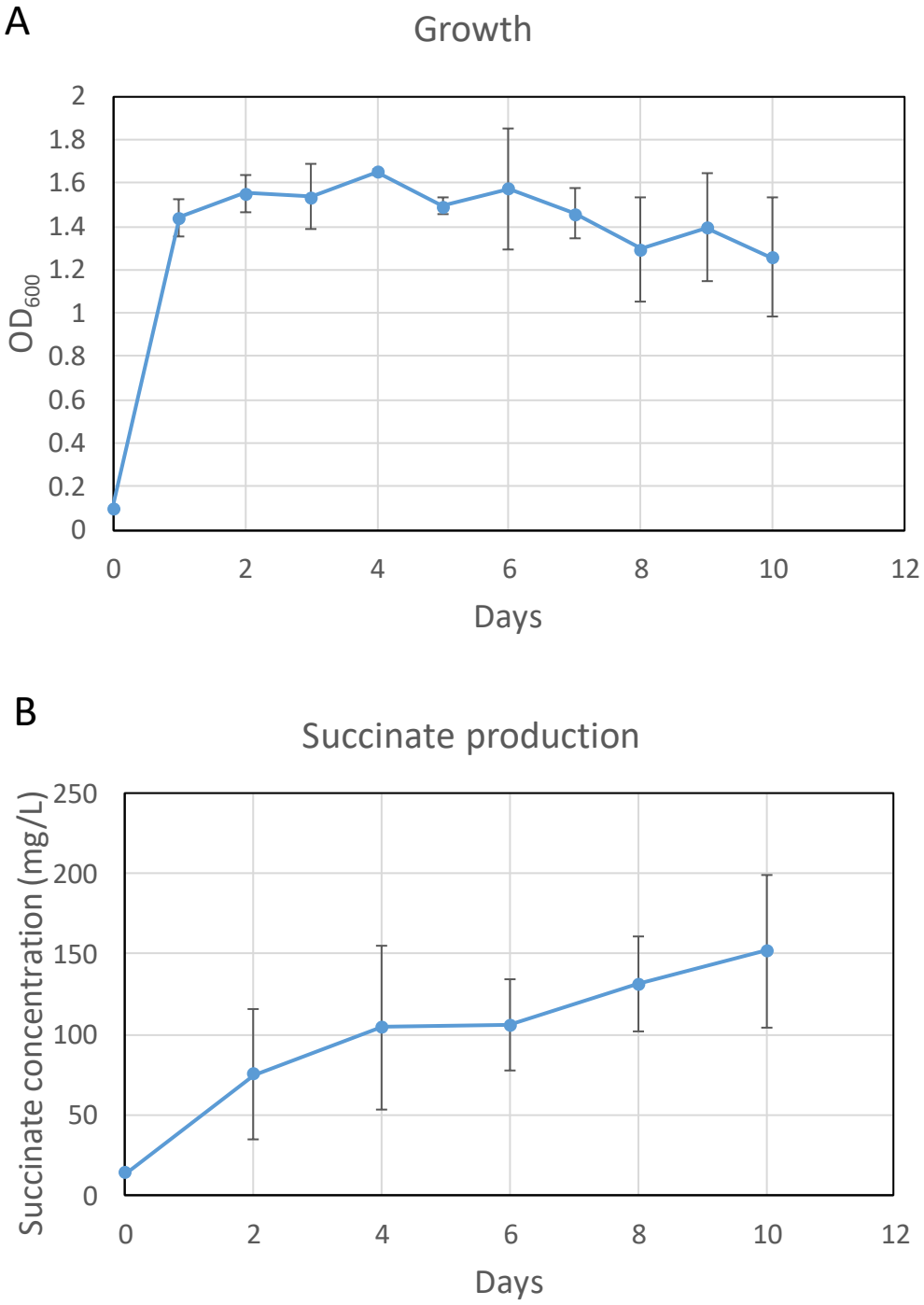


Figure 14. (A) Growth and (B) production of succinate by MGC6 harboring empty plasmid pHP13 cultivated in M2YG.

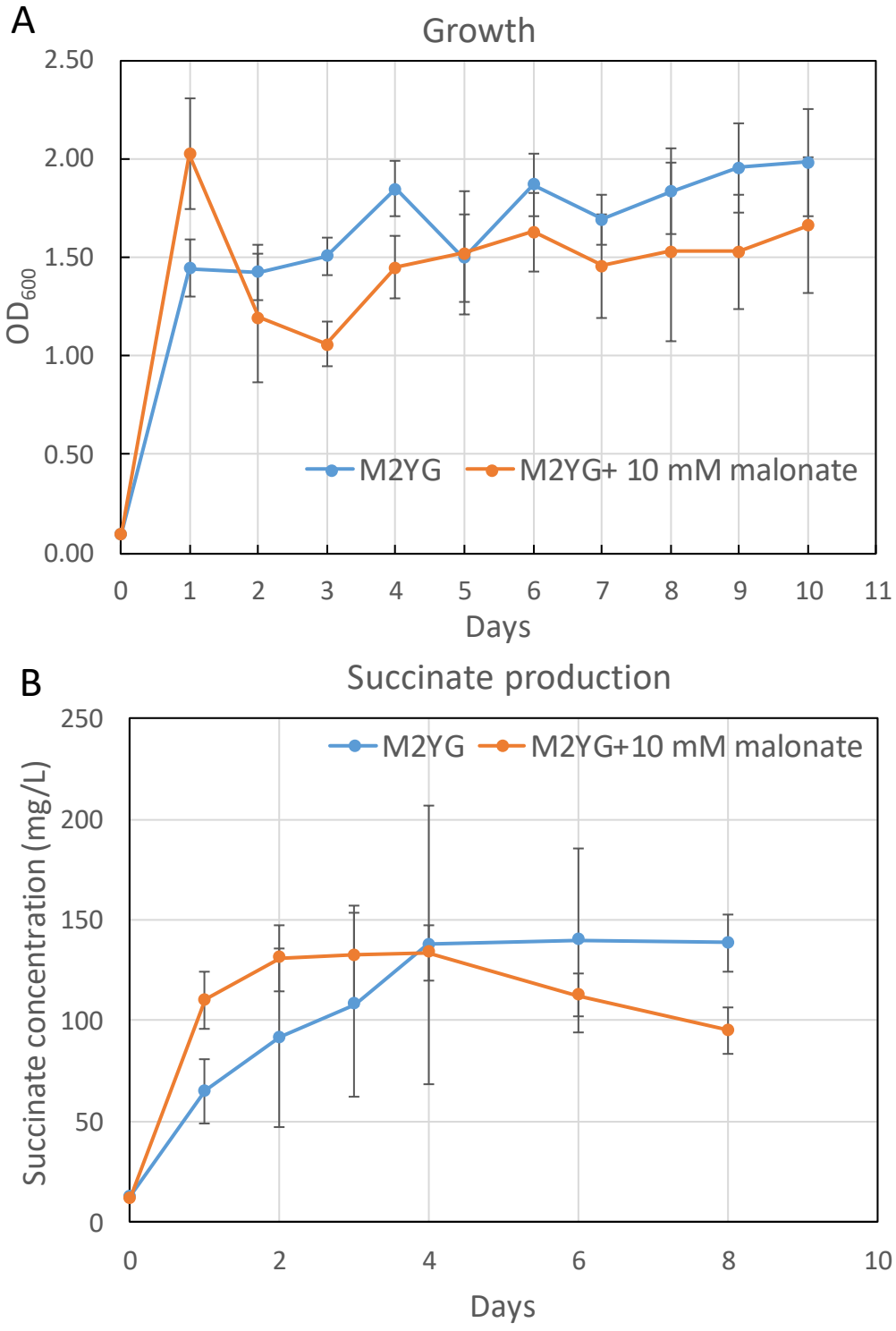


Figure 15. (A) Growth and (B) production of succinate by MGC6 harboring empty plasmid pHP13 cultivated in M2YG with and without malonate.

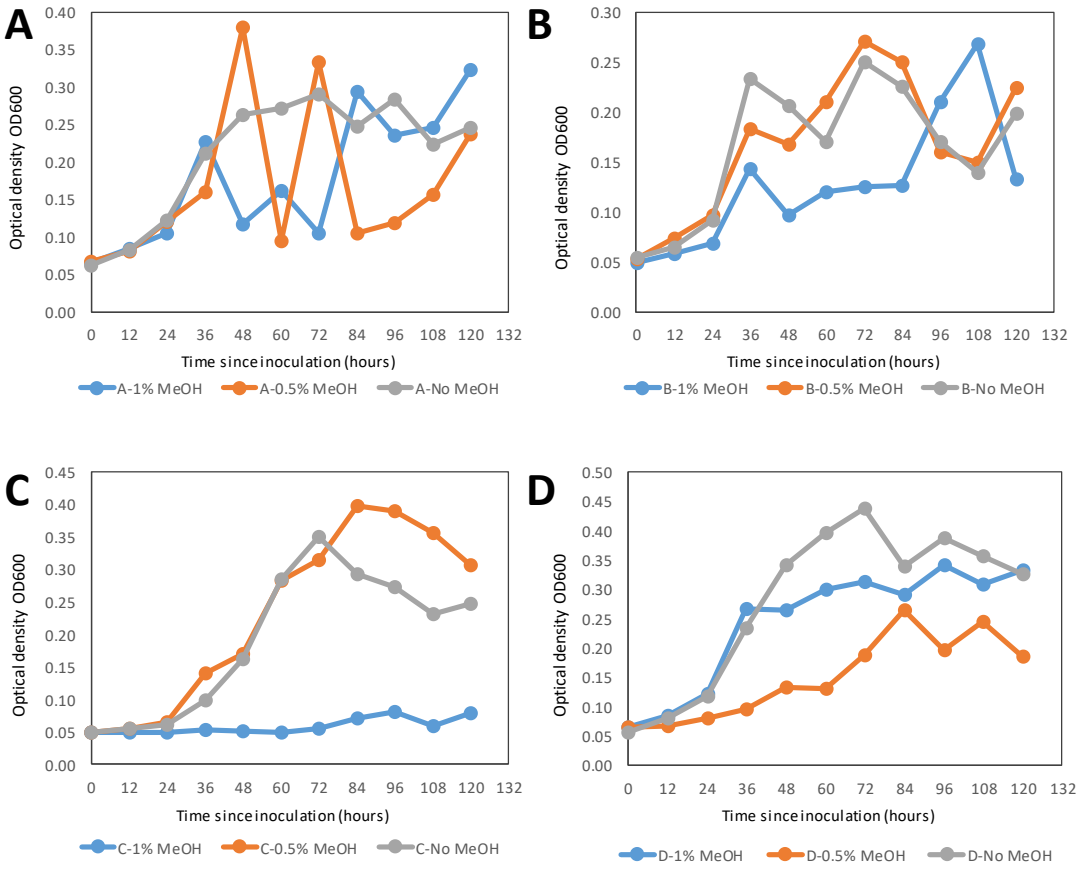


Figure 16. Growth of M. extorquens AM1 in nutrient broth containing either 0.5 or 1 % methanol. A) growth in glass screw cap flask inoculated with a loopful of cells. B) growth in testtube inoculated from a single colony. C) growth in glass screw cap flask inoculated from a single colony. D) growth in plastic baffled flask inoculated with a loolful of cells.

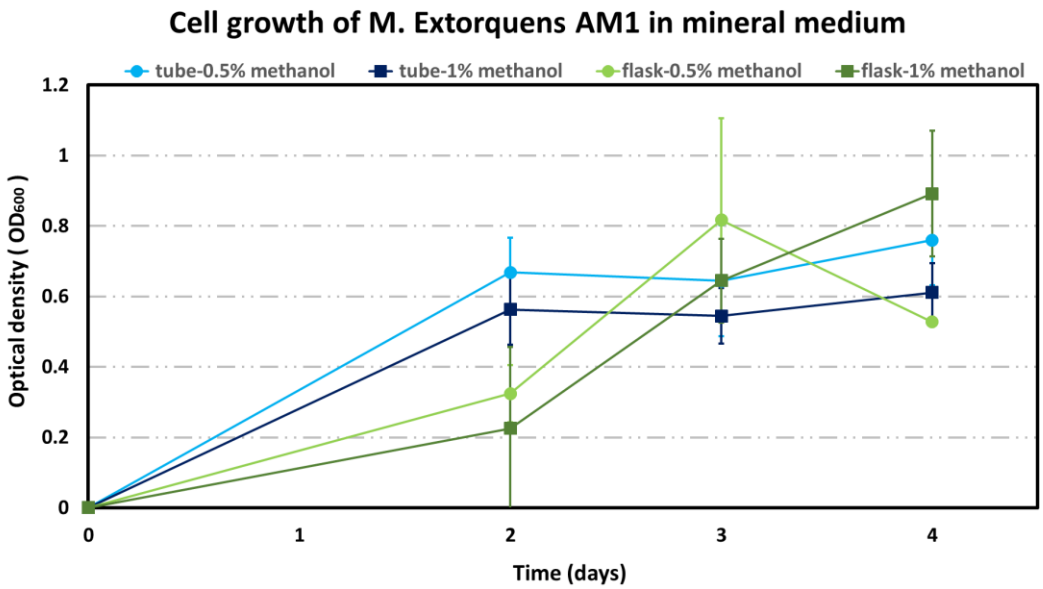


Figure 17. Growth of M. extorquens AM1 in mineral medium with either tube (4 ml) or flask (20 ml) containing different initial amounts of methanol.

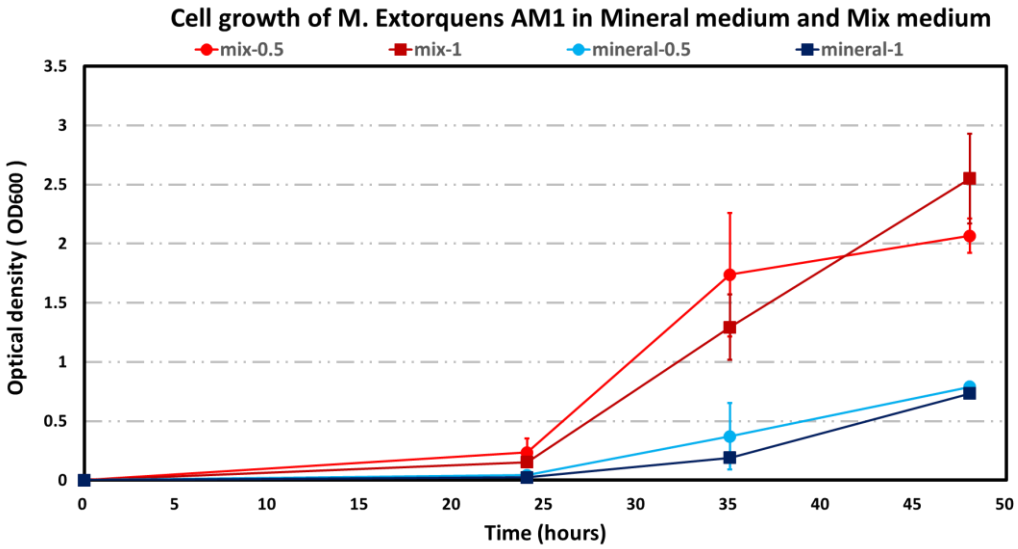


Figure 18. Growth of M. extorquens AM1 in test tubes using either mineral medium or mixed medium containing different initial amounts of methanol.

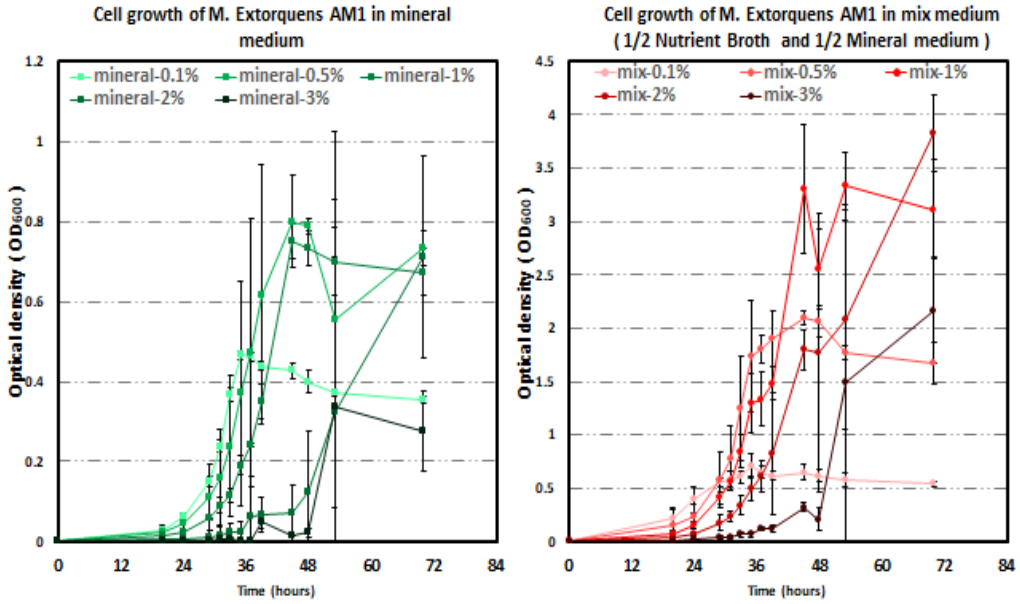
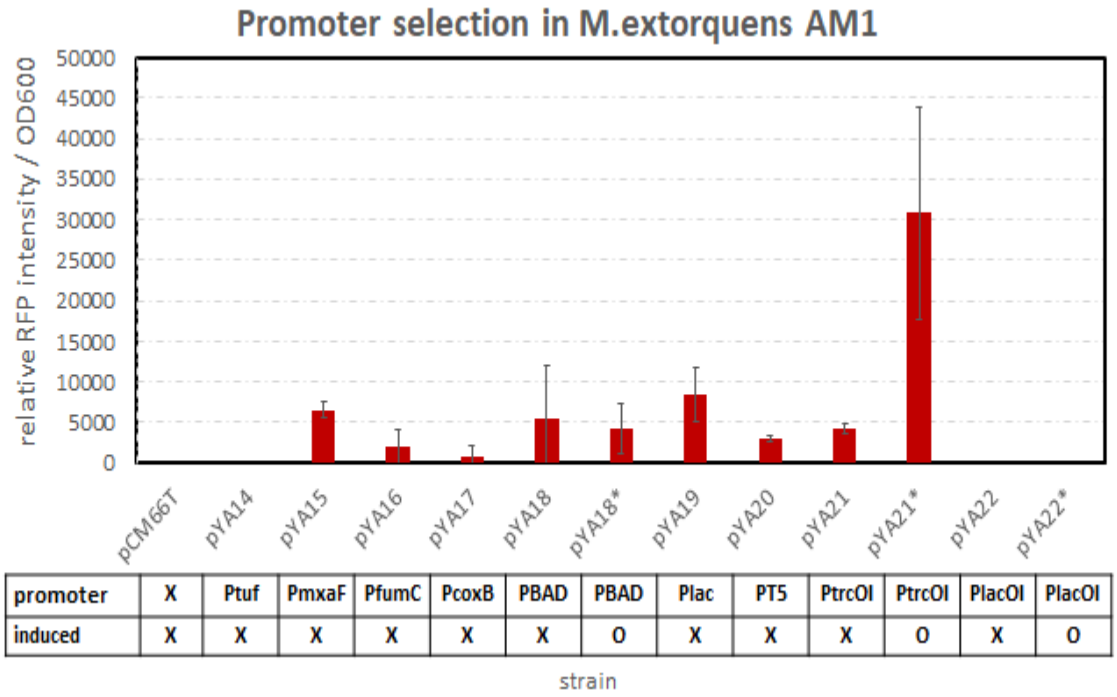


Figure 19. Growth of M. extorquens AM1 in test tubes using either mineral medium or mixed medium containing different initial amounts of methanol.



promoter	X	Ptuf	PmxaF	PfumC	PcoxB	PBAD	PBAD	Plac	PT5	PtrcOl	PtrcOl	PlacOl	PlacOl
induced	X	X	X	X	X	X	O	X	X	X	O	X	O

Figure 20. Expression of *rfp* to assess promoter strengths.

Table1. Methanol-based chemical production

Chemical target	Host organism	Titer (g/L)	Production system	Reference
Humulene	*Methylobacterium extorquens* (recombinant)	1.65	fed-batch	[15]
Mesaconate & methylsuccinate	*Methylobacterium extorquens* (recombinant)	0.65	flask batch	[14]
Glutamate	*Bacillus methanolicus* MGA3	55	fed-batch	[9]
Lysine	*Bacillus methanolicus* 13A52-8A66 (mutant)	35	fed-batch	[8]
Threonine	*Methylobacillus glycogenes* (mutant)	11	fed-batch	[11]
Serine	*Methylobacterium extorquens*	11.3	immobilized batch	[10]
1-Butanol	*Methylobacterium extorquens* (recombinant)	0.015	flask batch	[12]
Cadaverine	*Bacillus methanolicus* (recombinant)	11.3	fed-batch	[13]

Table 2. Plasmid list

PLASNID	GENOTYPE
PHP13	Broad host gram positive shuttle vector; cmR
PAH008	Pmdh::*ace*B *ace*A ; Cm^R
PAH009	Pmdh::*ace*B *ace*A *pck*A ; Cm^R
PAH013	Pmdh::*ace*B *ace*A *pyc* ; Cm^R
PAH014	Pmdh::*ace*B *ace*A *mae*A ; Cm^R
PTC11	Pmdh::*glt*A ; Cm^R
PTC12	Pmdh::*glt*A *ppc*^{Te} ; Cm^R
PTC13	Pmdh::*glt*A *ppc*^{Te} *acn* ; Cm^R
PJT-S	Pfba::gltA; Cm^R
PJT-T	Pfba::gltA, ppc; Cm^R
PJT-U	Pfba::gltA, ppc, acn; Cm^R
PCM66T	pCM66T
PYA14	pCM66T , Ptuf::rfp , Kan^R
PYA15	pCM66T , PmxaF::rfp , Kan^R
PYA16	pCM66T , PfumC::rfp , Kan^R
PYA17	pCM66T , PcoxB::rfp , Kan^R
PYA18	pCM66T , PBAD::rfp , Kan^R
PYA19	pCM66T , Plac::rfp , Kan^R
PYA20	pCM66T , PT5::rfp , Kan^R
PYA21	pCM66T , PtrcOI::rfp , Kan^R
PYA22	pCM66T , PlacOI::rfp , Kan^R
PYA24	pCM66T , PmxaF::dCas9-Ptuf::sgRNA(sdhA-4) , Kan^R
PYA25	pCM66T , PmxaF::dCas9-Ptuf::sgRNA(sdhA-7) , Kan^R
PYA26	pCM66T , PmxaF::dCas9-Ptuf::sgRNA(sdhB-4) , Kan^R
PYA27	pCM66T , PmxaF::dCas9-Ptuf::sgRNA(sdhB-18) , Kan^R
PYA28	pCM66T , PmxaF::dCas9-Ptuf::sgRNA(sdhC-5) , Kan^R
PYA29	pCM66T , PmxaF::dCas9-Ptuf::sgRNA(sdhC-44) , Kan^R
PYA30	pCM66T , PmxaF::dCas9-Ptuf::sgRNA(sdhD-17) , Kan^R
PYA31	pCM66T , PmxaF::dCas9-Ptuf::sgRNA(sdhD-55) , Kan^R
STRAIN	GENOTYPE
PB1	Wildtype PB1 strain of Bacillus methanolicus
MGC6	Isolated mutant from acridine orange treatment of Bacillus methanolicus strain MGA3 harboring a pNW33N plasmid
AM1	Wildtype Methylobacterium extorquens AM1