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Original Research Article

Characterization and directed evolution of propionyl-CoA carboxylase and its application in succinate biosynthetic pathway with two CO_2 fixation reactions

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ABSTRACT

Propionyl-CoA carboxylase (PCC) is a promising enzyme in the fields of biological CO_2 utilization, synthesis of natrual products, and so on. The activity and substrate specificity of PCC are dependent on its key subunit carboxyltransferase (CT). To obtain PCC with high enzyme activity, seven *pccB* genes encoding CT subunit from diverse microorganisms were expressed in recombinant *E. coli*, and PccB from *Bacillus subtilis* showed the highest activity *in vitro*. To further optimize this protein using directed evolution, a genetic screening system based on oxaloacetate availability was designed to enrich the active variants of $PccB_{Bs}$. Four amino acid substitutions (D46G, L97Q, N220I and I391T) proved of great assistance in $PccB_{Bs}$ activity improvement, and a double mutant of $PccB_{Bs}$ (N220I/I391T) showed a 94-fold increase of overall catalytic efficiency indicated by k_{cat}/K_m. Moreover, this $PccB_{Bs}$ double mutant was applied in construction of new succinate biosynthetic pathway. This new pathway produces succinate from acetyl-CoA with fixation of two CO_2 molecules, which was confirmed by isotope labeling experiment with $NaH^{13}CO_3$. Compared with previous succinate production based on carboxylation of phosphoenolpyruvate or pyruvate, this new pathway showed some advantages including higher CO_2 fixation potentiality and availability under aerobic conditions. In summary, this study developed a PCC with high enzyme activity which can be widely used in biotechnology field, and also demonstrated the feasibility of new succinate biosynthetic pathway with two CO_2 fixation reactions.

1. Introduction

CO_2 is released into the atmosphere massively due to the burning of fossil fuels, resulting in greenhouse effect and worldwide climate changes (Mohan et al., 2016). As reported by the International Energy Agency, the global CO_2 emissions caused by anthropogenic activities in 2017 was 32.5 gigatonnes, which has reached a historic high level and is expected to increase to 50 gigatonnes by 2050 (Shi et al., 2019). Therefore, reducing the net release of CO_2 into atmosphere has become an urgent task. To achieve this goal, one approach is converting CO_2 into multi-carbon molecules like fuels and other value-added chemicals by engineered biological systems (Siegel et al., 2015). It will not only decrease the CO_2 emission, but also provide free carbon source for the biosynthesis of renewable fuels and materials, representing a win-win strategy.

Up to date, there are six natural CO_2-fixation pathways reported: the Calvin cycle (Stitt et al., 2010), the 3-hydroxypropionate (3HP) cycle (Strauss and Fuchs, 1993), the reductive tricarboxylic acid (TCA) cycle (Evans et al., 1966), the 3-hydroxypropionate-4-hydroxybutyrate (3HP/4HB) cycle (Berg et al., 2007), the reductive acetyl-CoA pathway (Ljungdahl and Wood, 1969), and the dicarboxylate/4-hydroxybutyrate (DC/4HB) cycle (Huber et al., 2008). Each CO_2-fixation pathway has its own characteristics which determine the scope of application. The key enzyme RubisCO in Calvin cycle shows

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a strong side reaction with oxygen, which leads to the loss of fixed carbon (Walker et al., 2016). Because of the presence of oxygen-sensitive enzymes, the reductive TCA cycle, the reductive acetyl-CoA pathway and the DC/4HB cycle are anaerobic pathways (Ducat and Silver, 2012). However, in order to be applied in general synthetic biology platforms, an ideal route for one-carbon utilization should function in a robust manner under both aerobic and anaerobic conditions (Bar-Even et al., 2010). Consequently, we paid attention to the 3HP cycle containing carboxylation reactions of acetyl-CoA and propionyl-CoA with bicarbonate, which is oxygen independent. Part of this cycle has been introduced into *Escherichia coli* recombinant strains aiming to fix CO_2 to produce high value chemicals, such as 3HP (Liu et al., 2016).

Propionyl-CoA carboxylase (PCC) catalyzes the carboxylation of propionyl-CoA with bicarbonate producing methylmalonyl-CoA, which is the fourth step in 3HP cycle. PCC belongs to a subgroup of biotin-dependent short chain acyl-CoA carboxylases, which also include acetyl-CoA carboxylase (ACC), 3-metylcrotonyl-CoA carboxylase and so on (Wongkittichote et al., 2017). Enzymes in this group share similar structure composed of biotin carboxyl carrier protein (BCCP) domain, biotin carboxylase domain (BC), and carboxyltransferase domain (CT), and utilize a covalently bound biotin as cofactor. In the catalytic process, biotin binds to the active site of BCCP domain, and the BC domain catalyzes the MgATP-dependent carboxylation on the N1 nitrogen of biotin. Then the carboxylbiotin is translocated to allow interaction with the CT domain, and the carboxyl group is transferred from biotin to the alpha-carbon of corresponding substrate acyl-CoA (Wongkittichote et al., 2017). The CT domain totally determines the specificity of substrate, and can work with BCCP and BC domains of different enzyme (Diacovich et al., 2002; Lombard and Moreira, 2011; Tong, 2013).

Besides functioning in carbon fixation, PCC also plays an important role in the synthesis of secondary metabolites of microorganism, such as the biosynthesis of FK506 (Huang et al., 2013) and erythromycin (Tsai et al., 2001), as well as in the degradation of various amino acids (Gygax et al., 1984). The deficient activity of propionyl-CoA carboxylase in human body would cause deterioration of metabolic decompensation, propionic acidemia, hyperglycinemia or death (Deodato et al., 2006).

In this study, seven CT subunits of PCC from diverse bacterial species were expressed and purified in recombinant *E. coli*. Along with BCCP and BC subunits of *E. coli* ACC, PCC activity was determined *in vitro*. The PccB protein from *Bacillus subtilis* presented the highest enzyme activity among these seven proteins, and was further improved using directed evolution. Substitution of two amino acid residues (N220I/I391T) effected a 94-fold increase of overall catalytic efficiency indicated by k_{cat}/K_m. Furthermore, this $PccB_{Bs}$ mutant was applied in construction of new succinate biosynthetic pathway, which produces succinate from one acetyl-CoA and two CO_2 molecules.

2. Materials and methods

2.1. Strains and chemicals

The strains and plasmids used in this study were listed in Supplementary Table 1. The primers used in this study were synthesized by TSINGKE (Beijing, China), and listed in Supplementary Table 2. *E. coli* DH5α was used as the host strain to construct and store all recombinant plasmids, and *E. coli* BL21(DE3) was used for succinate biosynthesis and protein expression. The $NaH^{13}CO_3$ (99%) was purchased from Cambridge Isotope Laboratories (USA), propionyl-CoA and methylmalonyl-CoA were purchased from Sigma-Aldrich (St. Louis, MO, USA), and other chemicals were purchased from Aladdin (Shanghai, China) or Sinopharm (Beijing, China) of analytical grade or higher quality. PrimeSTAR Max DNA Polymerase (Takara, Dalian, China) was used for PCR. Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) was used for bacteria construction and protein expression, and minimal medium containing 14.04 g/L $K_2HPO_4{\cdot}3H_2O$, 5.24 g/L KH_2PO_4, 1 g/L NH_4Cl, 1 g/L NaCl, 0.5 g/L $MgSO_4$, 0.0037 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.0029 g/L $ZnSO_4{\cdot}7H_2O$, 0.0247 g/L H_3BO_3, 0.0025 g/L $CuSO_4{\cdot}5H_2O$, and 0.0158 g/L $MnCl_2{\cdot}4H_2O$, pH 7.0 was used for library screening and cultivation in 250-mL fermentor. Chloramphenicol (50 mg/L), kanamycin (50 mg/L), ampicillin (100 mg/L) and spectinomycin (50 mg/L) was used where appropriate.

2.2. Construction of plasmids and strains

Molecular cloning was performed using standard protocols (Green and Sambrook, 2012). The genes encoding AccA (accession No. CAQ30699.1), AccB (accession No. CAQ32718.1), AccC (accession No. CAQ33581.1), AccD (accession No. CAQ33582.1), and SucCD (accession No. CAQ31193.1 and CAQ31194.1) from *E. coli*, MCR (accession No. AAS20429.1), PCS (accession No. AAL47820.2), and MmcEM (accession No. ACL23899.1 and ACM54095.1) from *Chloroflexus aurantacus*, MCT complex (accession No. CBL57376.1, CBL57379.1 and CBL57378.1) from *Propionibacterium freudenreichii*, PccB from *Bacillus subtilis* (accession No. CAB14323.2), PccB from *Streptomyces coelicolor* (accession No. CAD30916.1), PccB from *Chloroflexus aggregans* (accession No. ACL25480.1), PccB from *Dehalococcoides mccartyi* (accession No. AGG06086.1), PccB from *Myxococcus xanthus* (accession No. ABF88262.1), PccB from *Sinorhizobium meliloti* (accession No. PTD27045.1), and PccB from *Sulfolobus tokodaii* (accession No. BAK54342.1) were amplified or synthesized by BGI Beijing (China), and cloned into appropriate vectors, respectively. The CRISPR-Cas9 system was used to realize the markerless gene deletion of *ygfG* gene from BL21 (DE3) chromosome as described previously (Jiang et al., 2015).

2.3. Directed evolution of $PccB_{BS}$

E. coli BL21 (DE3) chromosomal genes *mdh, ppc,* and *pck* were knocked out via P1 vir-mediated transduction as previously described (Moore, 2011), resulting strain Q3440. The donor strain JW3205, JW3928, and JW3366 were purchased from the Keio collection (Baba et al., 2006). The *accBC* genes were amplified from pACYC-*accADBC-pcs*, and cloned into pACYC-*mct* (synthesized by BGI Beijing, China) to generate pACYC-*accBC-mct*. The $pccB_{Bs}$ gene was amplified from pET-*mcrN-mcrC-*$pccB_{Bs}$*-mmcEM-sucCD* and cloned into vector pETDuet1 to generate pET-$pccB_{Bs}$.

Random mutagenesis of $pccB_{Bs}$ gene was carried out using error-prone PCR. The error-prone PCR system contains 20 mM Tris-HCl (pH = 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 3 mM $MgSO_4$, 0.3 mM $MnCl_2$, 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 2.5 units *EasyTaq* DNA polymerase, 0.2 mM 2279 and 2280 primers, 1 ng/μL plasmid pET-$pccB_{Bs}$ as template. The mutated $pccB_{Bs}$ fragments were inserted into pETDuet-1 and then transformed into *E. coli* DH5α. Transformants were plated on LB agar plate containing ampicillin and incubated at 37 °C overnight. The total number of colonies were counted, 23 colonies were randomly selected from the library for colony PCR to determine the insertion rate, and 11 positive colonies were subjected to nucleotide sequencing to determine the mutation rate of $pccB_{Bs}$ gene.

The mutant library was co-transformed with pACYC-*accBC-mct* into Q3440, and then they were grown on minimal medium plates supplemented with 0.025 g/L yeast extract, 0.05 g/L tryptone, 10 g/L glucose, 1 g/L sodium propionate, 4 g/L $NaHCO_3$, 40 mg/L biotin, and 100 μM IPTG. After cultured at 30 °C for 24 h, large clones were picked out and transferred to the liquid minimal medium. Strains were selected according to the OD_{600} level and $PccB_{BS}$ activity measured using whole cell extract.

Site-directed mutation of $pccB_{BS}$ was constructed using the Fast Site-Directed Mutagenesis Kit according to the manufacturer's instruction (TIANGEN, Beijing, China), and conformed using DNA sequencing.

2.4. Purification of recombinant proteins and enzyme activity assay

E. coli BL21(DE3) strains carrying expression plasmid were grown in

LB medium at 30 °C and the cells were induced with 100 μM IPTG at OD_{600} 0.6. His_6-tagged protein was purified using Ni-NTA His·Bind Column (Novagen) according to the manufacturer's instruction. 12% SDS-PAGE and Coomassie Blue staining were used to analyze the purified proteins.

The enzyme reaction mixture (100 μL) contained 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 10 mM KCl, 40 mM $NaHCO_3$, 1 mM Biotin, 6 mM ATP, 1 mM propionyl-CoA, 5 μM purified AccB protein, 5 μM purified AccC protein, and 0.5 μM purified PccB protein. The reaction was started by the addition of propionyl-CoA and was performed at 37 °C for 2 min. Then, the reaction mixture was mixed with 50 μL of 200 mM formic acid, and centrifuged at 15,000 rpm at 4 °C for 15 min. The supernatant was analyzed using HPLC-MSMS (Compact Q-TOF, Bruker) in the positive-ion mode. The ion chromatograms of propionyl-CoA, and methylmalonyl-CoA were extracted using the m/z of 824 ± 0.2 and 868 ± 0.2 respectively.

To determine the optimal temperature, the reactions were incubated at a series of temperatures (27, 32, 37, and 42 °C) at pH 7.5. To determine the optimal pH, Tris-HCl buffers with different pH (pH 6.5, 7.0, 7.5, 8.0, and 8.5) were tested at 37 °C. To determine the K_m and k_{cat} values, a series of propionyl-CoA concentrations (25, 33, 40, 50, 100, and 500 μM) were used and the reactions were carried out at 37 °C at pH 7.5. The K_m and k_{cat} values were determined through Lineweaver-Burk plot (Cleland, 1979).

2.5. Fermentation and succinate production

The strain was grown overnight in LB broth and 1:100 diluted into Mini Bioreactor (250 mL, Applikon, Netherlands) containing 100 mL minimal medium supplemented with 20 g/L glucose. The temperature was kept at 37 °C, the pH was controlled at 7.0 via automatic addition of 10% (v/v) ammonia water, and stirring rate was set to 400 rpm. When the OD_{600} reached about 5, the temperature was switched to 30 °C and 100 μM IPTG, 4 g/L $NaHCO_3$, 40 mg/L biotin, 7 mg/L vitamin B12 were added to the culture. 4 g/L $NaHCO_3$, 40 mg/L biotin were supplemented every 12 h and the fermentation process carried on for 48 h. The succinate concentration in culture supernatant was determined using Agilent 1200 Infinity HPLC system with an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad).

In ^{13}C labeling experiments, $NaHCO_3$ was replaced by $NaH^{13}CO_3$. Succinate was purified from the culture supernatant using a Waters 2545 Preparative HPLC system with an Xterra Prep RP18 column (7.8 × 150 mm), and analyzed using Compact Q-TOF (Bruker) in the negative-ion mode. The natural isotope distribution of succinate was calculated at the Scientific Instrument Services website (www.sisweb.com/mstools/isotope.htm).

3. Results and discussion

3.1. Purification and activity assay of PCC from diverse bacteria

To obtain PCC enzyme with high enzymatic activity, *pccB* genes encoding CT subunit of PCC from diverse bacterial species were synthesized and cloned into the empty vector pETDuet-1 fused with an N-terminal His_6-tag, respectively. Among them, PccBs from *S. coelicolor* and *M. xanthus* were experimentally identified (Kimura et al., 1997; Rodriguez and Gramajo, 1999), the one from *C. aggregans* shared 98% amino acid sequence identity with proved PccB protein from *Chloroflexus aurantiacus* (Klatt et al., 2007), and the others were annotated as putative PccB based on the computational evidences. When expressed in *E. coli* BL21(DE3), each protein was observed as a distinct band with expected molecular weight on SDS-PAGE, demonstrating those genes were properly cloned and expressed in recombinant strain. Then the proteins were purified by Ni-affinity chromatography (Fig. 2A). In addition, His_6-tagged AccB and AccC from *E. coli*, the BCCP and BC subunits of ACC, were also purified for activity assay along with CT subunits from different species, instead of their own BCCP and BC subunits.

In previous studies, PCC activity was usually determined by three methods. First, PCC was assayed in terms of the incorporation of radioactive HCO_3^- into acid-stable material (Diacovich et al., 2002; Hunaiti and Kolattukudy, 1982), which brought the drawbacks of using radioisotope materials, like radiation safety and waste disposal. Second, it could be measured following the release of ADP and phosphate (Diacovich et al., 2002; Soriano et al., 2006). Furthermore, the product methylmalonyl-CoA could be quantitated by HPLC with UV detector (Shimazu et al., 2004). However, the fixation of radioactive HCO_3^- and the hydrolysis of ATP both happen in the reaction catalyzed by BC subunit (Fig. 1), hard to determine accurately the activity of key subunit CT, and HPLC has poor selectivity of different CoA esters.

To develop an alternative method for assaying PCC activity, we carried out the quantitation of methylmalonyl-CoA using HPLC-MSMS. The mass spectrum provided structural information to distinguish product methylmalonyl-CoA from substrate propionyl-CoA, and the ion chromatograms of methylmalonyl-CoA extracted with the m/z of 868 ± 0.2 were used to calculate its concentration according to a standard curve. This method showed good linearity and selectivity.

In an *in vitro* reaction containing Mg^{2+}, ATP, bicarbonate, biotin, purified PccB from *B. subtilis* and AccBC from *E. coli*, methylmalonyl-CoA was detected as a specific peak with a retention time identical to that of standard (Fig. 2B). Furthermore, dissociation of the ion of 868.0 Da led to other masses such as 428.0, 361.1, 317.1, and 259.0 Da, exactly the same with methlymalonyl-CoA standard (Fig. 2B). However, negative control without addition of purified CT subunit was deficient in methylmalonyl-CoA production (data not shown). These results demonstrated that the purified PCC subunits could assemble *in vitro* and catalyze the carboxylation of propionyl-CoA with bicarbonate successfully. Next, seven CT subunits were measured *in vitro* along with AccBC protein from *E. coli*. All these CT subunits showed propionyl-CoA carboxytransferase activity, and the PccB protein from *B. subtilis* presented the highest activity of 1.79 ± 0.13 μmol/min/mg protein (Fig. 2C), which was subjected to laboratory evolution.

3.2. Directed evolution of PccB from B. subtilis

Directed evolution has been proved as a powerful vehicle to engineer proteins for desirable properties (Jackel et al., 2008). To mimic natural evolution, iterative cycles of gene mutagenesis and selection of desired mutants are carried out during the evolution process. High-throughput screening method is required to sort out the rare but useful mutants from the complex library.

To enrich active variants of PccB protein, a genetic selection system was constructed relied on the oxaloacetate availability. There are four genes, *ppc*, *mdh*, *pck* and *aspC*, involved in the oxaloacetate biosynthesis in *E. coli*. It was planned to knock out all those four genes to generate a strain deficient in the biosynthesis of oxaloacetate (Fig. 3A). However, a triple mutant of *E. coli* BL21(DE3) (Q3440, *E. coli* BL21(DE3) Δ*ppc* Δ*pck* Δ*mdh*) has lost the growth capacity in minimal medium beforehand (Fig. 3B). Then, a complementary pathway for oxaloacetate biosynthesis was constructed in plasmids pACYC-*accBC-mct* and pET-$pccB_{Bs}$. The methylmalonyl-CoA carboxyltransferase (MCT) from *P. freudenreichii* can transfer a carboxyl group from methylmalonyl-CoA to pyruvate to yield propionyl-CoA and oxaloacetate (Thornton et al., 1993). An active PccB protein will produce methylmalonyl-CoA as the substrate of MCT and enable preferential growth in minimal medium. Thus, the activity of PccB was linked to the growth rate of host strain in minimal medium. As shown in (Fig. 3B), coexpression of $PccB_{Bs}$ and MCT in this strain significantly enhanced the cell density. The strain Q3440/pACYC-*accBC-mct* could grow slightly probably because that *E. coli* endogenous ACC could also catalyze the propionyl-CoA carboxylation with a much lower activity (Tong, 2013). These results proved the effectiveness of this genetic selection system.

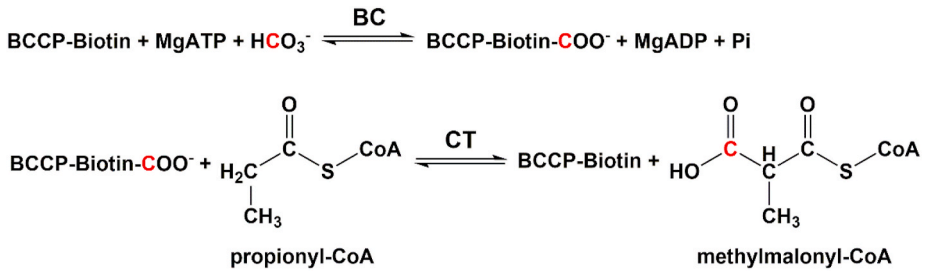


Fig. 1. The reaction catalyzed by PCC. BCCP, biotin carboxyl carrier protein; BC, biotin carboxylase; CT, carboxyltransferase.

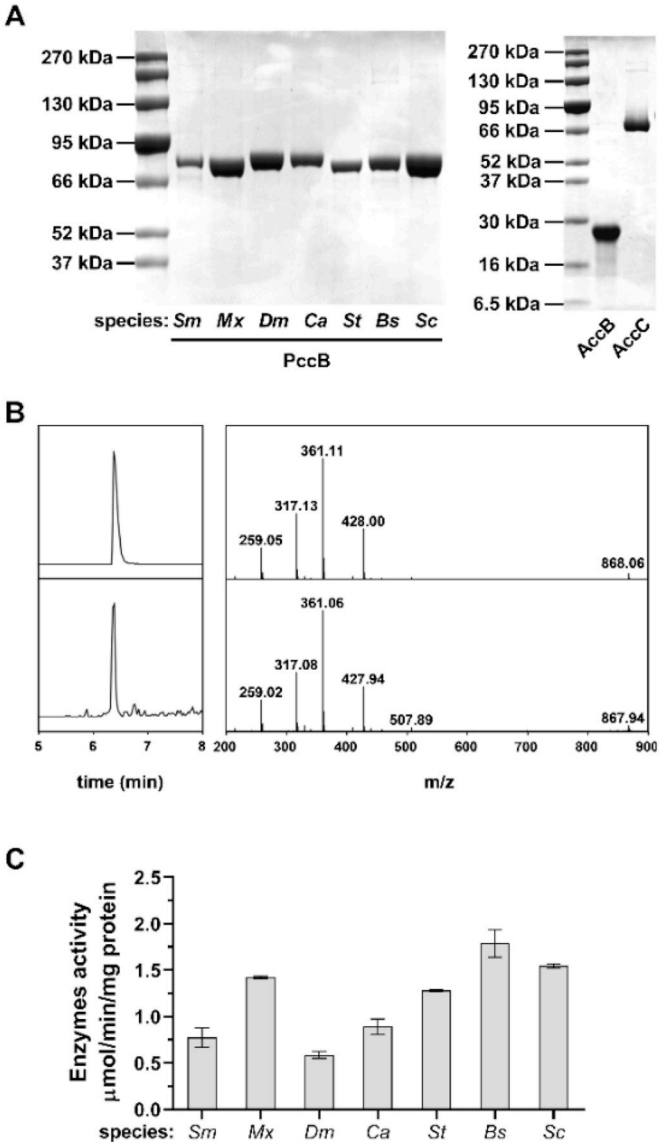


Fig. 2. Purification and activity assay of the propionyl-CoA carboxytransferase subunits from diverse bacteria species along with *E. coli* AccBC. (A) SDS-PAGE of purified PccB proteins and AccBC. (B) HPLC-MSMS analysis of methylmalonyl-CoA standard (up panel) and *in vitro* reaction containing PccB from *B. subtilis* (bottom panel). Both extracted ion chromatography (left section) and mass spectrometry (right section) of methylmalonyl-CoA were shown. (C) Enzyme activity comparison of PccB proteins from diverse bacterial species. *Sm*, *S. meliloti*; *Mx*, *M. xanthus*; *Dm*, *D. mccartyi*; *Ca*, *C. aggregans*; *St*, *S. coelicolor*; *Bs*, *B. subtilis*; *Sc*, *S. tokodaii*. Data represent mean ± standard deviation (n = 2).

A random mutant library of $pccB_{Bs}$ gene was constructed using error-prone PCR, which contained about 4.5×10^4 clones. The insertion rate of this library was 82.6% (19 positive out of 23 random colonies), and the mutation rate of $pccB_{Bs}$ gene was 1.13‰ (19 mutations in 11 sequenced clones). After screen using the genetic selection system, 28 colonies were selected out and whole-cell lysate from each colony was used in the PCC activity assay. Seven mutants presented enzymatic activity at least 1.5-time higher than that of wild-type $PccB_{Bs}$ protein, and DNA sequencing revealed that all these mutants encoded one or two amino acid substitutions (Fig. 4A). Then twelve single mutants of $PccB_{Bs}$ protein were constructed by site-directed mutagenesis to figure out the contribution of each substitution to the increase of enzyme activity. Following protein purification and *in vitro* activity assay, four amino acid substitutions (D46G, L97Q, N220I and I391T) proved of great assistance in $PccB_{Bs}$ activity improvement (Fig. 4B). To further optimize this protein, combinations of these four mutations were constructed. Surprisingly, only five mutants with multiple amino acid substitutions presented higher enzymatic activity when compared with wild-type $PccB_{BS}$ protein, and a double mutant N220I/I391T showed the highest activity of 10.05 ± 0.51 μmol/min/mg protein (Fig. 4C).

The kinetic properties of $PccB_{Bs}$ wild-type protein and N220I/I391T mutant with the highest activity were determined *in vitro*. For both $PccB_{Bs}$ protein and N220I/I391T mutant, the optimal temperature for activity was 37 °C and the optimal pH was 7.5 (Fig. 5A and B), and the original $PccB_{Bs}$ protein seemed to be more resistant to temperature than the mutant (Fig. 5A). The original and evolved $PccB_{Bs}$ proteins both obey the Michaelis-Menten Kinetics, and the parameters were determined through Lineweaver-Burk plot. The K_m value for propionyl-CoA of original $PccB_{Bs}$ protein was 745 ± 45 μM, 2.8-time higher than that of N220I/I391T mutant, indicating that the substrate affinity of $PccB_{Bs}$ protein was dramatically enhanced during the evolution process. The k_{cat} values of wild-type and mutant $PccB_{Bs}$ proteins were 0.4 ± 0.03 s^{-1} and 13.3 ± 0.11 s^{-1}, respectively, suggesting that the reaction was more favorable towards methylmalonyl-CoA production with $PccB_{Bs}$ mutant. The overall catalytic efficiency, which is represented by k_{cat}/K_m, increased 94-fold for the N220I/I391T mutant when compared with the original $PccB_{Bs}$ protein (Fig. 5C).

3.3. Succinate biosynthesis with two CO_2 fixation reactions

3HP cycle is a promising CO_2-fixation pathway containing two carboxylation reactions of short-chain acyl-CoA with bicarbonate, which has been partially expressed in *E. coli* to produce 3HP (Liu et al., 2016). Based on previous study, we further cloned several genes involved in 3HP cycle to construct succinate biosynthetic pathway which fixes two CO_2 molecules (Fig. 6A). Propionyl-CoA synthase (PCS) converts 3HP into propionyl-CoA, which is carboxylated by PCC to generate (*S*)-methylmalonyl-CoA. Then, (*S*)-methylmalonyl-CoA is transformed into its isomer succinyl-CoA by methylmalonyl-CoA epimerase (MmcE) and methylmalonyl-CoA mutase (MmcM). Finally, succinyl-CoA is deesterificated by succinyl-CoA synthetase (SucCD). Through this pathway, succinate is synthesized from one acetyl-CoA and two CO_2 molecules. In the resultant strain Q3120, genes encoding

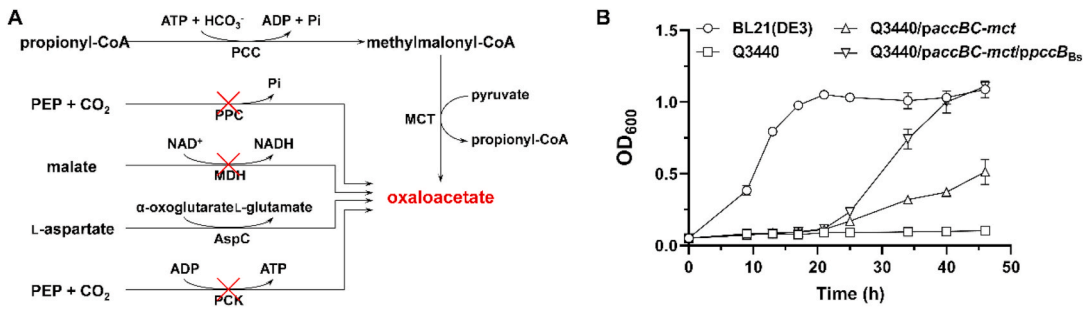


Fig. 3. Genetic selection system relied on the oxaloacetate availability to enrich the active mutants of $PccB_{Bs}$ protein. (A) Complementary pathway for oxaloacetate biosynthesis constructed with $PccB_{Bs}$, AccBC and methylmalonyl-CoA carboxyltransferase MCT. (B) Growth of *E. coli* strains with and without alternative oxaloacetate biosynthetic pathway. The strains were inoculated into shaking flasks containing minimal medium in triplicate. Data represent mean ± standard deviation (n = 2).

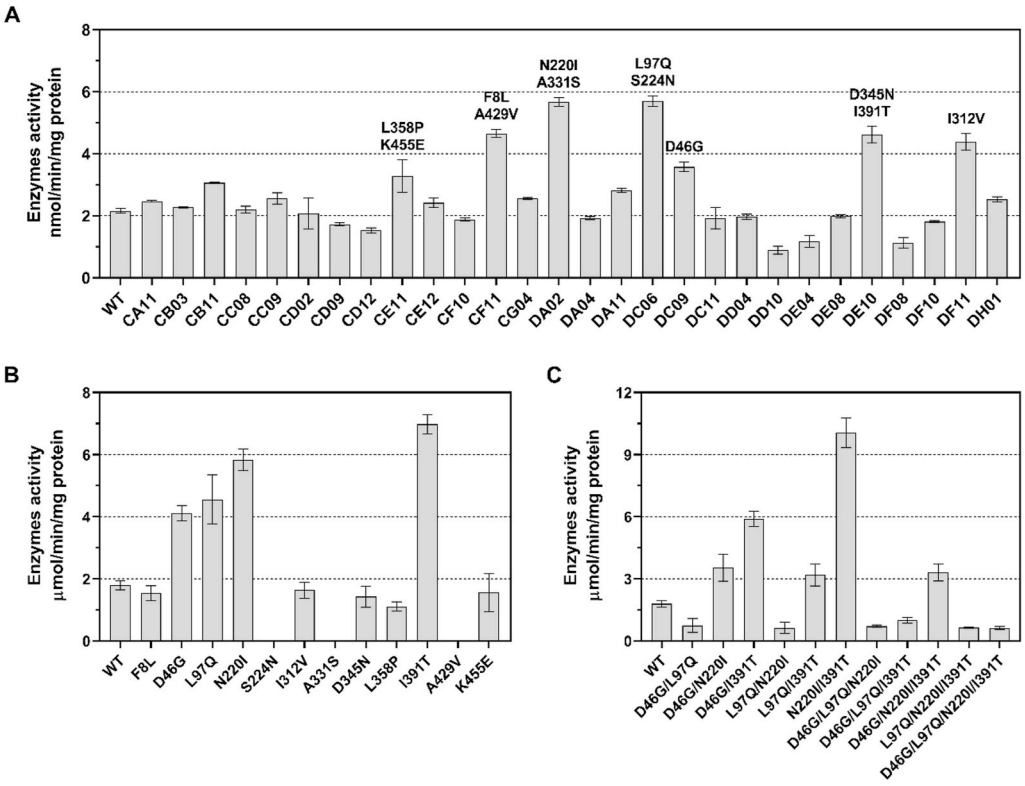
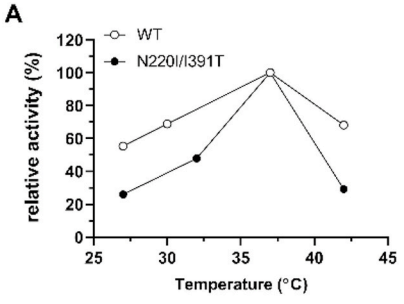
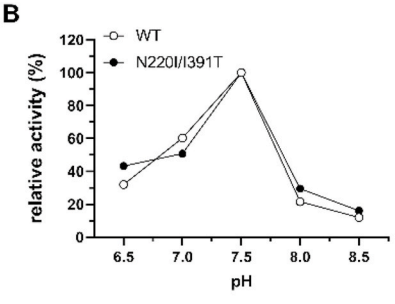


Fig. 4. *In vitro* activity assay of $PccB_{Bs}$ mutants. (A) PCC activity tested using whole-cell lysates from 28 colonies selected in library screening. Enzyme activity of seven mutants was more than 1.5 times higher than that of wild-type $PccB_{Bs}$ protein, and corresponding amino acid substitutions were shown. (B) PCC activity determined using purified $PccB_{Bs}$ mutants with single amino acid substitution. (C) PCC activity determined using purified $PccB_{Bs}$ mutants with multiple amino acid substitutions. Data represent mean ± standard deviation (n = 2 in Fig. 4A, and n = 3 in Fig. 4B and C).

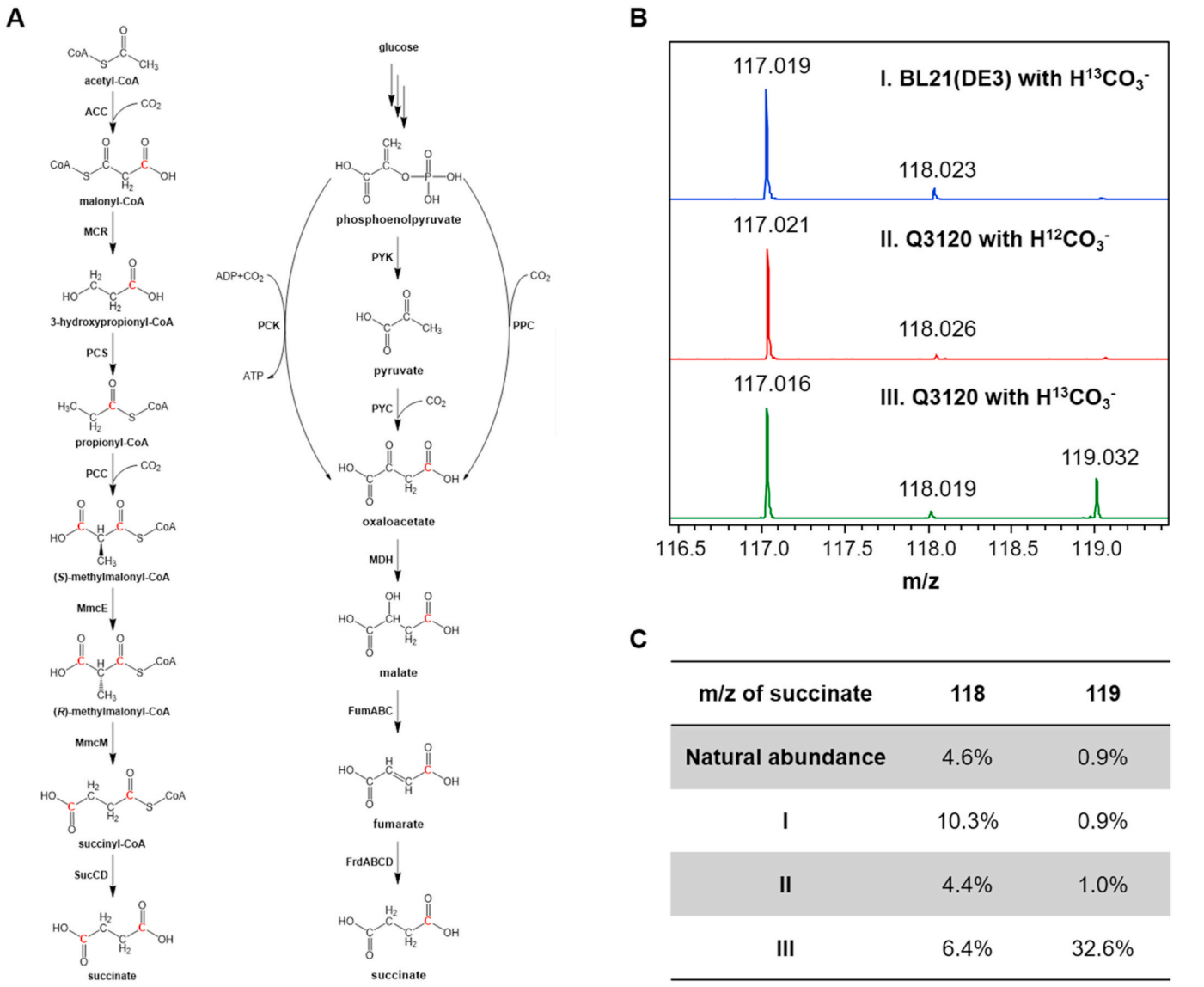




C

	WT	N220I/I391T
Enzyme activity (μmol/min/mg protein)	1.79 ± 0.13	10.05 ± 0.51
K_{cat} (s^{-1})	0.4 ± 0.03	13.3 ± 0.11
K_m (μM)	745 ± 45	267 ± 11
K_{cat}/K_m (mM^{-1} s^{-1})	0.53	49.9

Fig. 5. Kinetic analysis of $PccB_{Bs}$ wild-type protein and N220I/I391T mutant. (A) Effects of temperature on enzyme activity of purified $PccB_{Bs}$ wild-type protein and N220I/I391T mutant. The reactions were performed at pH 7.5. (B) Effects of pH on enzyme activity of $PccB_{Bs}$ wild-type protein and N220I/I391T mutant. The reactions were performed at 37 °C. For wild-type $PccB_{Bs}$, 100% corresponds to the enzyme activity of 1.79 ± 0.13 μmol/min/mg protein at 37 °C and pH 7.5. For N220I/I391T mutant, 100% corresponds to the enzyme activity of 10.05 ± 0.51 μmol/min/mg protein at 37 °C and pH 7.5. (C) Kinetic properties of $PccB_{Bs}$ wild-type protein and N220I/I391T mutant. Data represent mean ± standard deviation (n = 3).



C

m/z of succinate	118	119
Natural abundance	4.6%	0.9%
I	10.3%	0.9%
II	4.4%	1.0%
III	6.4%	32.6%

Fig. 6. Construction and validation of succinate biosynthetic pathway with two CO_2 fixation reactions. (A) Comparison of the succinate biosynthetic pathway constructed in this study and its traditional pathway. ACC, acetyl-CoA carboxylase; MCR, malonyl-CoA reductase; PCC, propionyl-CoA carboxylase; MmcE, methylmalonyl-CoA epimerase; MmcM, methylmalonyl-CoA mutase; SucCD, succinyl-CoA synthetase; PYK, pyruvate kinase; PCK, PEP carboxykinase; PPC, PEP carboxylase; PYC, pyruvate carboxylase; MDH, malate dehydrogenase; FumABC, fumarate hydratase; FrdABCD, fumarate reductase. The carbon atoms from CO_2 were highlighted in red. (B) Mass spectrum of succinate produced by *E. coli* BL21(DE3) wild-type strain and Q3120 with ^{12}C- or ^{13}C-labled HCO_3^-. (C) Percentage of succinate with different molecular weight produced by *E. coli* BL21(DE3) wild-type strain and Q3120 with ^{12}C- or ^{13}C-labled HCO_3^-. (B) and (C) show a representative result from two independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

acetyl-CoA carboxylase complex and PCS were cloned into the plasmid vector pACYCDuet-1, and genes *mcr*, $pccB_{Bs}$, *mmcE*, *mmcM* and *sucCD* were cloned into the vector pETDuet-1.

Succinate is a key metabolic intermediate of the TCA cycle, and can also be produced relying on the carboxylation of phosphoenolpyruvate (PEP) under anaerobic conditions in *E. coli* (Tan et al., 2013), resulting in fixation of one CO_2 molecule along with production of one succinate molecule (Fig. 6A). To distinguish whether the succinate is synthesized through the heterologous pathway we constructed, *E. coli* BL21(DE3) wild-type and Q3120 strain were cultured aerobically in minimal medium supplemented with $H^{13}CO_3^-$ or $H^{12}CO_3^-$. Then succinate was purified from fermentation broth using preparative HPLC and analyzed by mass spectrometer in the negative-ion mode (Fig. 6B). Succinate has a molecular weight of 118, and will present a m/z of 117 if without ^{13}C atom. The m/z of 118 indicates that one ^{13}C atom is incorporated into the succinate molecule, and it should be synthesized via PEP carboxylation. The m/z of 119 suggests that this succinate molecule contains two ^{13}C atoms and is produced through our pathway.

The natural abundance of succinate with molecular weight of 119 and 120 was 4.6% and 0.9%, respectively. When BL21(DE3) wild-type strain was supplied with $H^{13}CO_3^-$, succinate with the m/z of 118 and 119 accounted for 10.3% and 0.9% of the total production of succinate (Fig. 6B and C), indicating that under this condition majority of succinate was produced by BL21(DE3) strain through TCA cycle and minor from PEP carboxylation. When the recombinant strain Q3120 was supplied with $H^{12}CO_3^-$, the distribution of succinate with different m/z was similar with the natural abundance (Fig. 6B and C). When Q3120 strain was fed with $H^{13}CO_3^-$, the content of succinate with the m/z of 119 significantly increased to 32.6%, while succinate with the m/z of 118 only increased slightly (Fig. 6B and C), demonstrating that the pathway

constructed here is feasible to synthesize succinate from acetyl-CoA with fixation of two CO_2 molecules, and responsible for production of most succinate under this condition.

In 250-mL bioreactor, the strain Q3120 accumulated 1.04 ± 0.09 g/L succinate after 48 h of aerobic fed-batch fermentation. As the 3HP production using acetyl-CoA carboxylase (ACC) and malonyl-CoA reductase (MCR) has been optimized thoroughly (Liu et al., 2013, 2016), further improvement was carried out with emphasis on the conversion from 3HP to succinate using the following strategies: (1) The $PccB_{Bs}$ protein was replaced by its double mutant N220I/I391T with the highest enzyme activity. (2) The *ygfG* gene encoding methylmalonyl-CoA decarboxylase was knocked out to block consumption of methylmalonyl-CoA. (3) The genes *mmcEM* or *sucCD* were not overexpressed to reduce the metabolic burden of recombinant strain, as *E. coli* has endogenous genes with similar functions (Dayem et al., 2002; Evans et al., 1993; Nishimura, 1986). As shown in Fig. 7A, each strategy effected an increase of succinate production in different degree, and combination of these three strategies resulted in the highest succinate production of 2.66 ± 0.26 g/L, which was achieved by the strain Q3124.

The effect of plasmid copy number on succinate production was also investigated. In strain Q3124, genes *mcr*, $pccB_{Bs}$, and *mmcEM* were cloned into the vector pETDuet-1, which contains a pBR322 origin. The DNA fragment carrying these genes and their regulatory elements were cleaved from the pETDuet-1 based plasmid, and cloned into three different plasmids: the low copy number plasmid pKD46 with a pSC101 origin, ColA origin-derived vector pCOLADuet-1, and high copy number plasmid pUC19 with a pMB1 origin. The resultant plasmids were transformed into *E. coli* BL21(DE3) Δ*ygfG* strain along with pACYC-*accADBC-pcs* to generate strains Q3175, Q3360, and Q3173, respectively. Under aerobic fed-batch conditions in 250-mL bioreactor, strain Q3124 presented the highest succinate production of 2.66 ± 0.26 g/L with a yield of 0.08 g/g glucose, demonstrating a CO_2 fixation efficiency of 0.94 mmol/L/h (Fig. 7B).

Here, a succinate biosynthetic pathway with two CO_2 fixation reactions was constructed and optimized in recombinant *E. coli*. Succinate is an important platform chemical, ranked as one of top 12 value-added biobased chemicals (Werpy and Petersen, 2004). Previously, succinate was usually produced through the carboxylation of PEP or pyruvate under anaerobic conditions (Fig. 6A) (Tan et al., 2013; Wang et al., 2011; Zhang et al., 2010). Comparatively speaking, our pathway has some expected advantages. First, this route shows higher potentiality in CO_2 fixation. Succinate is synthesized from acetyl-CoA and CO_2, and half of carbon atoms in succinate is derived from CO_2, while only one CO_2 fixation reaction, carboxylation of PEP or pyruvate, is employed in the traditional succinate biosynthetic pathway. Secondly, succinate production dependent on previous pathways required the strict anaerobic conditions, while this new succinate pathway can function under aerobic conditions.

Despite the progress already made in new succinate pathway, there are still some problems unsolved. For example, only 2.66 ± 0.26 g/L succinate was produced under fed-batch conditions with the CO_2 fixation efficiency of 0.94 mmol/L/h, and further development is required to achieve a high succinate production. Targeted metabolome analysis should be carried out to determine the intracellular concentration of key intermediates including propionyl-CoA, 3-hydroxypropionyl-CoA, methylmalonyl-CoA and succinyl-CoA, and this information will help us to identify the limiting step of this pathway and direct the further optimization of our recombinant strain. Furthermore, the enzyme expression level in a multistep pathway is not simply "the more the better", and functional balance between enzymes in this pathway should also be considered (Liu et al., 2016). Another problem is that plenty of ATP and reducing power are consumed for succinate synthesis from acetyl-CoA and CO_2, and a promising solution is feeding electricity to bacteria to produce intracellular energy and reducing equivalents (Chen et al., 2018). In addition, glucose was used as carbon source in this study, and CO_2 was released in the conversion of pyruvate into acetyl-CoA, which reduced the conversion efficiency of glucose to succinate. Using some carbon sources which can be metabolized to acetyl-CoA without CO_2 release such as fatty acids, ethanol or acetate should be an atom-economic way (Liu et al., 2019; Xu et al., 2017; Sun et al., 2020).

4. Conclusion

PCC is a promising enzyme, which can be used in biological CO_2 utilization and synthesis of natrual products. In order to obtain PCC with high enzyme activity, seven *pccB* genes encoding CT subunit from diverse microorganisms were expressed in recombinant *E. coli*, and PccB from *B. subtilis* showed the highest activity in activity assay *in vitro*. Moreover, directed evolution was used to further enhance the activity of $PccB_{Bs}$, and substitution of two amino acid residues (N220I/I391T) effected a 94-fold increase of overall catalytic efficiency indicated by k_{cat}/K_m. In addition, this $PccB_{Bs}$ mutant was applied in construction of new succinate biosynthetic pathway, which produces succinate from one acetyl-CoA and two CO_2 molecules. Compared with previous succinate production based on carboxylation of PEP or pyruvate, this new pathway proved of higher CO_2 fixation efficiency and availability under

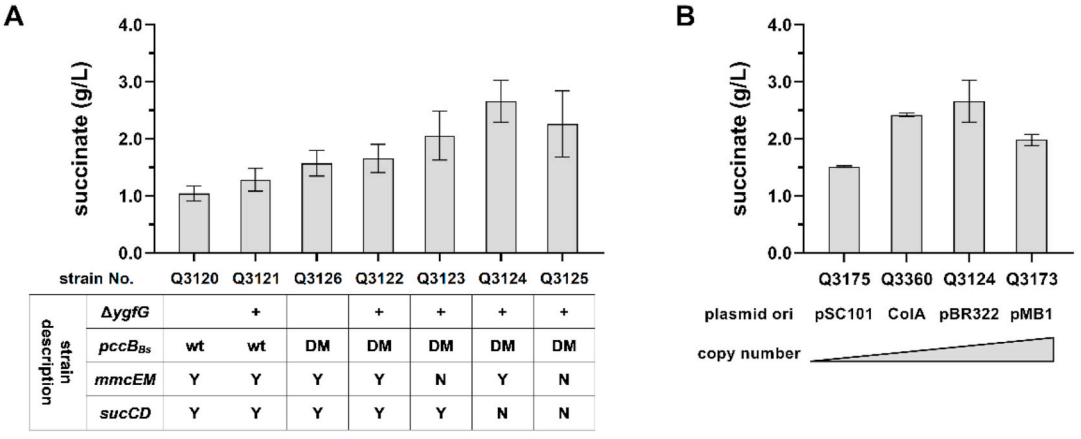


Fig. 7. Optimization of succinate producing strain. (A) Effect of different genes on succinate production. The detailed strain description was shown in the bottom form. For Δ*ygfG*, "+" means that the gene *ygfG* was knocked out in the corresponding strain. For $pccB_{Bs}$, "wt" represents wild-type $pccB_{Bs}$ gene, and "DM" represents the double mutant $pccB_{Bs}$ N220I/I391T. For *mmcEM* and *sucCD*, "Y" means that those genes were overexpressed in the corresponding strain, and "N" means no overexpression. (B) Effect of plasmid copy number on succinate production. Data represent mean ± standard deviation (n = 2).

aerobic conditions. In future, this pathway can be further developed on succinate productivity, supply of energy and reducing equivalence, and conversion efficiency of carbon source.

CRediT authorship contribution statement

Xiutao Liu: Investigation, Writing - original draft, Methodology, Formal analysis. **Xinjun Feng:** Conceptualization, Project administration, Funding acquisition. **Yamei Ding:** Investigation, Visualization. **Wenjie Gao:** Methodology, Investigation, Formal analysis. **Mo Xian:** Resources, Writing - review & editing, Supervision, Funding acquisition. **Jichao Wang:** Writing - review & editing, Data curation. **Guang Zhao:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

This work has been included in patent applications by Qingdao Institute of Bioenergy and Bioprocess Technology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2020.08.012.

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