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Metabolic engineering of *Propionibacterium freudenreichii*: effect of expressing phosphoenolpyruvate carboxylase on propionic acid production

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Abstract Propionic acid is currently produced mainly via petrochemicals, but there is increasing interest in its fermentative production from renewable biomass. However, the current propionic acid fermentation process suffers from low product yield and productivity. In this work, the gene encoding phosphoenolpyruvate carboxylase (PPC) was cloned from *Escherichia coli* and expressed in *Propionibacterium freudenreichii*. PPC catalyzes the conversion of phosphoenolpyruvate to oxaloacetate with the fixation of one CO_2. Its expression in *P. freudenreichii* showed profound effects on propionic acid fermentation. Compared to the wild type, the mutant expressing the *ppc* gene grew significantly faster, consumed more glycerol, and produced propionate to a higher final titer at a faster rate. The mutant also produced significantly more propionate from glucose under elevated CO_2 partial pressure. These effects could be attributed to increased CO_2 fixation and resulting changes in the flux distributions in the dicarboxylic acid pathway.

Keywords Propionibacteria · Propionic acid · Phosphoenolpyruvate carboxylase · Glycerol · *Propionibacterium freudenreichii* · CO_2 fixation

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Introduction

Propionic acid is commonly used as a food preservative and as a chemical intermediate in various industrial processes for the manufacturing of polymers, pesticides, and pharmaceuticals (Boyaval and Corre 1995; Playne 1985). Currently, propionic acid is mainly produced from petrochemicals which are non-renewable and increasingly costly due to the rising oil prices. These, in addition to the rising demands for natural ingredients in consumer products, have led to interests in fermentative production of propionic acid from bio-renewable resources (Dishisha et al. 2013; Kagliwal et al. 2013; Liu et al. 2012; Wang and Yang 2013). However, much work is still required to make fermentative propionic acid production competitive to petrochemical processes. Propionibacteria are gram-positive bacteria that can synthesize propionic acid through the dicarboxylic acid pathway with acetate and succinate as two main byproducts. Byproduct formation and the inhibition effect of propionic acid on cells are two major concerns that have ignited continuous research efforts aiming to enhance propionic acid fermentation with higher final product concentration, yield, and productivity (Coral et al. 2008; Himmi et al. 2000; Suwannakham and Yang 2005; Zhang and Yang 2009a; Zhu et al. 2012). However, most of the previous studies focused on process optimization and strain development through conventional mutagenesis and adaptation. To date, little has been done on the metabolic engineering of propionibacteria for enhanced propionic acid production (Ammar et al. 2013; Suwannakham et al. 2006; Zhuge et al. 2013).

At the branch point of the dicarboxylic acid pathway of propionibacteria, pyruvate is converted to either acetyl-CoA, which leads to the formation of acetate, or oxaloacetate (OAA) via methylmalonyl-CoA carboxyltransferase (MMC, also named oxaloacetate transcarboxylase), which transfers

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one carbon from methylmalonyl-CoA to pyruvate to form OAA and propionyl-CoA, leading to the formation of propionate (see Fig. 1). Some propionibacteria can also use methylmalonyl-CoA decarboxylase (MMD) to decarboxylate methylmalonyl-CoA to propionyl-CoA and CO_2, which does not involve pyruvate, although MMD gene has not been found in the genome of *Propionibacterium freudenreichii* (Falentin et al. 2010; Horváth et al. 2012; Meurice et al. 2004; Parizzi et al. 2012; Vörös et al. 2012). Also, some propionibacteria, such as *Propionibacterium acidipropionici* but not *P. freudenreichii*, have pyruvate carboxylase (PYC), which can convert pyruvate to OAA with CO_2 fixation and the consumption of one ATP. Some microorganisms, such as *Escherichia coli* but not any known propionibacteria, can use phosphoenolpyruvate carboxylase (PPC) to convert phosphoenolpyruvate (PEP) to OAA by fixing CO_2. Theoretically, the flux to OAA, and thus propionate, can be increased by overexpressing PYC or PPC. Compared to PYC, directing carbon from PEP to OAA by PPC at an earlier point that bypasses the pyruvate branch point should have more prominent effect on propionate production. A mutant of *Corynebacterium glutamicum* with increased PPC activity has been reported to also have higher glutamic acid productivity (Sawada et al. 2010). In addition, overexpressing PPC has been proven to be effective for increasing succinic and fumaric acids production in *E. coli* and *Rhizopus oryzae*, respectively (Millard et al. 1996; Song et al. 2013; Zhang et al. 2012).

Therefore, we postulated that PPC can increase carbon flux towards OAA and propionate production in propionibacteria. The goal of this study was thus to express a heterologous *ppc* in propionibacteria and evaluate its effects on propionic acid production from glucose and glycerol. In this work, *ppc* from *E. coli* was successfully expressed in *P. freudenreichii* using the expression vector pKHEM04 (Kiatpapan et al. 2000; Kiatpapan and Murooka 2001, 2002) and the mutant showed increased cell growth and propionate production. To the best of our knowledge, this is the first report on enhancing propionic acid production by expressing *ppc* in propionibacteria.

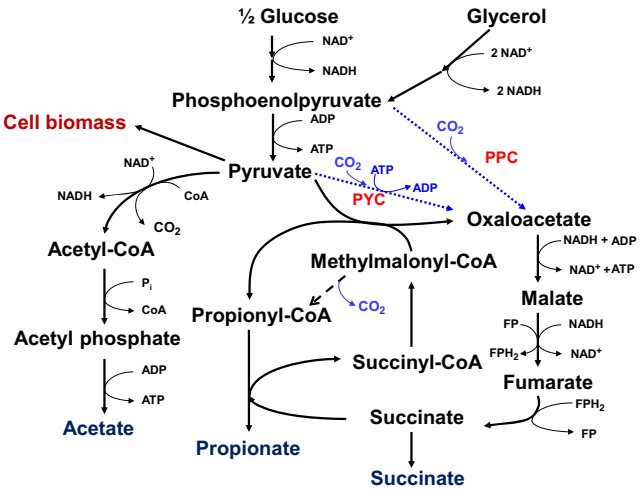

Fig. 1 Dicarboxylic acid pathway for propionic acid biosynthesis in propionibacteria. Some propionibacteria such as *P. acidipropionici* ATCC 4875 have pyruvate carboxylase (*PYC*) that can convert pyruvate to oxaloacetate with CO_2 fixation. The expression of heterologous phosphoenolpyruvate carboxylase (*PPC*) also allows the direct conversion of phosphoenolpyruvate to oxaloacetate with CO_2 fixation. Meanwhile, some propionibacteria has methylmalonyl-CoA decarboxylase (MMD) that can decarboxylate methylmalonyl-CoA to propionyl-CoA and CO_2

Materials and methods

Bacterial strains, plasmids, and media

Table 1 lists all bacterial strains and plasmids used in this work. Both *E. coli* DH5α and str. K12 substr. DH10B were grown aerobically in Luria-Bertani (LB) medium at 37 °C. For *E. coli* DH5α transformants, LB was supplemented with 100 μg/mL ampicillin. To prepare competent cells and for cell recovery after electroporation, propionibacteria were grown anaerobically in sodium lactate broth (NLB) containing 10 g/L sodium lactate, 10 g/L yeast extract, and 10 g/L trypticase soy broth at 32 °C. For fermentation kinetics studies, cells were grown anaerobically at 32 °C in the fermentation medium (10 g/L yeast extract, 5 g/L trypticase, 0.25 g/L K_2HPO_4, 0.05 g/L $MnSO_4$, 25–50 g/L carbon source, pH 6.5) supplemented with 2−4 % (*w*/*v*) $CaCO_3$ to buffer the pH during fermentation, unless otherwise noted. To study the effect of propionic acid on cell growth, media with different propionate concentrations (0, 5, 10, and 20 g/L) but without $CaCO_3$ were used. All media were autoclaved at 121 °C, 103.4 kPa for 30 min. To culture and maintain mutants, sterile hygromycin B was added to the autoclaved media to a final concentration of 250 μg/mL. Working cultures were kept at 4 °C for short-term storage, while the stock cultures were kept at −80 °C for long-term storage.

Construction of *ppc* expression vector and transformation

The expression vector (pKHEM04-*ppc*) was constructed from the shuttle vector pKHEM04 and *ppc* gene (accession no. ACB04967.1) amplified, using the primers shown in Table 1, from *E. coli* genome following similar procedures described by Ammar et al. (2013) (see Fig. S1 in Supplementary Materials). For verification, the cloned gene in the constructed vector was sequenced at the Plant-Microbe Genomic Facility at the Ohio State University. The transformation of propionibacteria with pKHEM04-*ppc* was done by electroporation following the procedures described elsewhere (Ammar et al. 2013). After electroporation, cells were grown on the NLB agar supplemented with 250 μg/mL hygromycin B for 7 to 10 days before colonies were picked from the plates. The

Table 1 Bacterial strains, plasmids, and primers used in this study

Strain/plasmid/primer	Relevant characteristics	Source/reference
Bacterial strains		
P. acidipropionici		
ATCC 4875	WT, not transformable with pKHEM04-*ppc*	ATCC
P. freudenreichii		
DSM 20271	WT, transformable with pKHEM04-*ppc*	DSM
DSM 4902	WT, transformable with pKHEM04-*ppc*	DSM
Pf(*ppc*)	DSM 20271 transformant of pKHEM04-*ppc*	This study
E. coli		
DH5α	Host cells for plasmids amplification	Invitrogen
K-12 substr. DH10B	DNA template for cloning *ppc*	Invitrogen
EC-pGEM-*ppc*	*E. coli* with plasmid pGEM-*ppc*	This study
EC-4P	*E. coli* with plasmid pKHEM04-*ppc*	This study
Plasmids		
pGEM-T	Cloning vector, Ap^r	Promega
pGEM-*ppc*	pGEM-T with *ppc*	This study
pKHEM04	Expression vector with p4 promoter, Ap^r, and Hyg^r	Kiatpapan and Murooka 2001
pKHEM04-*ppc*	pKHEM04 with *ppc*	This study
Primers		
ppc-F (forward primer)	gctagtccatggccatgaacgaacaatattccgcattgc	
ppc-R (reverse primer)	atgcagcatatgttattagccggtattacgcatacctg	

Ap^r ampicillin resistance gene, *Hyg^r* hygromycin B resistance gene, *WT* wild type

transformants were confirmed by PCR cloning using the *ppc* primers shown in Table 1.

PPC enzyme activity assay

To prepare the cell extract for activity assay, exponential-phase cells grown on NLB were harvested by centrifugation (7,000 rpm for 10 min), washed with ice-cold water three times, resuspended in 3 mL of ice-cold Tris-HCl buffer (25 mM, pH 7.4), and then treated with ultra-sonication using a sonic dismembrator (Fisher Scientific, Model 100) to disrupt cells, which included 20 cycles of 5-s ultra-sonication followed by 25 s standing on ice to avoid protein damage by heat. Protein concentration in cell extracts was determined by Bradford protein assay (Bio-Rad) in duplicate using bovine serum albumin as standard.

The PPC activity was measured following the procedures described by Zhang et al. (2012). Aliquots of cell extracts were added to the reaction mixture (0.1 M Tris-HCl buffer at pH 8.0, 0.01 M $MgCl_2$, 2.5 mM phosphoenolpyruvic acid, 0.2 mM NADH, 0.01 M $NaHCO_3$, and 5 units of malate dehydrogenase (Sigma-Aldrich), and the decrease in absorbance of NADH at 340 nm was monitored to determine the change in NADH with the extinction coefficient of 6.22/mM/cm and the amount of PPC. One activity unit is defined as the amount of PPC capable of oxidizing 1 μmol NADH per minute at 25 °C and pH 8.0.

Fermentation kinetics

Batch fermentations of glucose and glycerol were first studied in serum bottles under anaerobic conditions by purging fermentation media with nitrogen gas prior to autoclaving. Unless otherwise mentioned, freshly prepared cells grown on NLB to an OD_{600}~2.0 were used to inoculate the bottles at 5 % (*v*/*v*). Batch fermentations were also studied in 1-L stirred-tank bioreactors, each containing 0.5 L medium, at 32 °C, with pH controlled at 6.5 using 6 M NaOH and agitation at 100 rpm. The inoculum used was 25-mL freshly prepared cells in NLB to an OD_{600}~2.0. After inoculation, N_2 gas was sparged into the medium for ~30 min to purge oxygen from the bioreactor. Samples were collected at suitable time intervals to analyze for carbon source consumption and organic acids production.

Effects of CO_2 partial pressure

To evaluate the effects of CO_2 partial pressure on CO_2 fixation and propionic acid production, batch fermentations of glucose were studied in two sets of serum bottles, one tightly sealed to allow gas pressure buildup to ~2 atm (absolute pressure) measured with a pressure gauge and the other installed with a one-way check valve, which would release gas to the atmosphere and maintain the pressure at ~1 atm. The fermentation kinetics with and without CO_2 pressurization were studied in

media containing ~25 g/L glucose and 50 g/L $CaCO_3$ for pH buffering at ~5.0, with an initial seeding density of ~OD_{600} 50, for 70 h.

Analytical methods

Optical density as a measure of cell density was determined in a 1.5-mL cuvette (light path length = 1 cm) with a spectrophotometer (Shimazu, Columbia, MD, UV-16-1) at 600 nm. One OD_{600} unit was found to be equivalent to ~0.2 g/L cell dry weight. The concentrations of substrate (glucose or glycerol) and products (acetic, propionic, and succinic acids) in the fermentation broths were determined using high-performance liquid chromatography (HPLC) with an organic acid column (Bio-Rad, HPX-87H) following the procedures previously described by Suwannakham and Yang (2005).

Statistical analysis

All experiments were carried out in duplicate or triplicate, and the mean and standard error are reported. To assess the significance of different kinetic parameters, data was subject to Student's *t* test analysis with $p<0.05$ being significantly different.

Results

PCR cloning and expressing *ppc* in propionibacteria

The plasmid pKHEM04-*ppc* containing the *ppc* gene encoding PPC from *E. coli* str. K-12 substr. DH10B under the control of the p4 promoter from *P. freudenreichii* subsp. *shermanii* IFO12424 (Piao et al. 2004a) was constructed and used to transform several propionibacteria (*P. acidipropionici* ATCC 4875, *P. freudenreichii* DSM 20271, and *P. freudenreichii* DSM 4902). After electroporation, no colony from *P. acidipropionici* was obtained on the NLB agar plates with antibiotic (hygromycin B), whereas some colonies were obtained from the two *P. freudenreichii* strains (DSM 4902 and 20271). These colonies were picked and subcultured in NLB liquid media with hygromycin B to check their abilities to grow and produce propionic acid. Positive transformants were confirmed with the following: (1) positive culture PCR with *ppc* primers, (2) positive PCR with *ppc* primers and the plasmids isolated and purified from transformed colonies, and (3) positive PCR with *ppc* primers using plasmids extracted from *E. coli* which had been transformed with plasmids extracted from transformants earlier (data not shown). Finally, all positive transformants were screened against their corresponding wild-type (WT) cells for produced propionate and acetate titers in serum-bottle fermentations. The transformants from DSM 4902 produced less propionic acid while those from DSM 20271 generally gave better performance. The *P. freudenreichii* DSM 20271 mutant, namely, Pf(*ppc*), producing the highest level of propionate and best P/A ratio was further investigated to study the effects of expressing *ppc* on the PPC enzyme activity and the fermentation kinetics.

PPC enzyme activity

Based on the NADH consumption rate, the PPC activities in the cell extracts of Pf(*ppc*) and WT were estimated at 4.59±0.06 and 3.12±0.09 (U/mg), respectively. Compared to the WT, the mutant showed ~50 % higher specific enzyme activity, indicating that *ppc* from *E. coli* was expressed and functional in *P. freudenreichii* DSM 20271. It should be noted that the WT has no endogenous PPC (Falentin et al. 2010; Meurice et al. 2004) and the observed activity based on the NADH consumption was attributed to other reactions that might also consume NADH and thus should be regarded as background noise.

Effect of propionic acid on cell growth

To study the effect of *ppc* expression on cell growth, the WT and Pf(*ppc*) mutant cells were grown on glycerol in the presence of various amounts of propionic acid (0, 5, 10, and 20 g/L) and the results are shown in Fig. 2. Without propionic acid, Pf(*ppc*) showed a significantly higher specific growth rate (0.089±0.003 vs. 0.078±0.002/h) and grew to a higher final cell density (OD 4.7 vs. 3.7). However, the effect was not as pronounced in the presence of 5 g/L propionic acid (final OD 2.5 for mutant vs. 1.9 for WT) and diminished at the higher propionic acid concentrations of 10 and 20 g/L, which strongly inhibited cell growth in both cultures.

Batch fermentation with glucose as carbon source in serum bottle

Figure 3 shows the fermentation kinetics with *P. freudenreichii* DSM 20271 WT and the mutant expressing *ppc* gene, Pf(*ppc*), grown on glucose as the carbon source. Without $CaCO_3$ for buffering the pH, the mutant grew faster (0.102±0.001 vs. 0.095±0.001/h for the WT) and reached a higher OD_{600} of 6.8 (vs. 5.2 for the WT) in 100 h (Fig. 3a, b). Thereafter, both WT and mutant cell growth leveled off due to the drop in pH to below 4.5 because of the accumulating acids. Compared to the WT, the mutant Pf(*ppc*) also gave a slightly higher glucose consumption rate (0.046±0.003 vs. 0.040±0.001 g/L h) and a significantly higher propionate productivity (0.029±0.002 vs. 0.021±0.001 g/L h), although the propionate/acetate (*P*/*A*) ratio and acid yields were not significantly different (see Table 2). The fermentation kinetics was also studied with

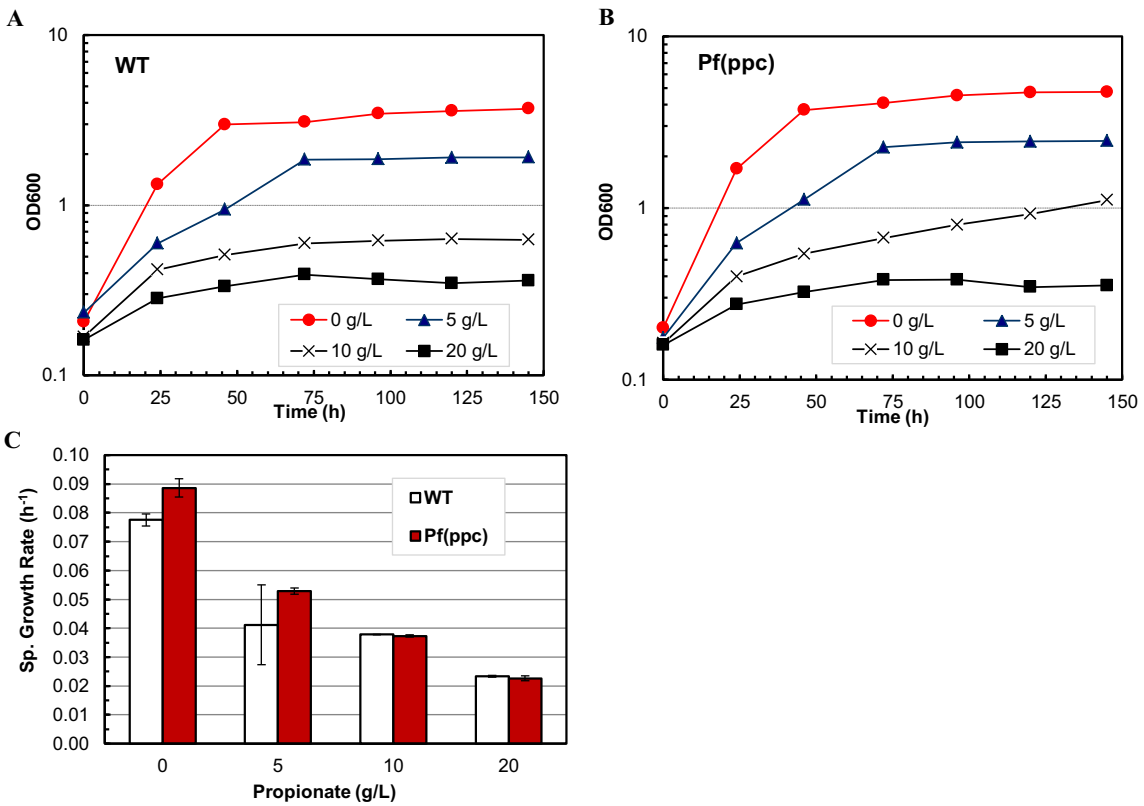

Fig. 2 Effect of propionic acid on cell growth on glycerol without $CaCO_3$ for pH buffering. The initial medium pH was ~6.5. **a** Growth kinetics of wild type (WT). b Growth kinetics of the mutant Pf(*ppc*) expressing *ppc*. **c** Comparison of specific growth rates of WT and Pf(*ppc*) at various propiononate concentrations

$CaCO_3$ to buffer the pH and neutralize the produced organic acids (Fig. 3c, d). Compared to the WT, the mutant Pf(*ppc*) had a much higher propionate productivity (0.066±0.006 vs. 0.046±0.001 g/L h) and glucose consumption rate (0.142± 0.013 vs. 0.090±0.020 g/L h). Interestingly, the fermentation with the mutant reached significantly higher final concentrations of propionate (14.9 vs. 10.7 g/L) and acetate (7.5 vs. 4.6 g/L). However, there was no significant difference in the propionic acid yield and the *P*/*A* ratio between the mutant and the WT (see Table 2). Nevertheless, a significantly higher propionate/succinate (*P*/*S*) ratio was obtained in the fermentation with the mutant as compared to the WT.

Batch fermentation with glycerol as carbon source in serum bottle

Figure 4 shows the fermentation kinetics of the WT and Pf(*ppc*) grown on glycerol as carbon source in serum bottles. In the absence of $CaCO_3$ for pH buffering, the mutant grew faster (0.089±0.002 vs. 0.078±0.003/h), consumed glycerol much faster (0.053±0.004 vs. 0.038±0.002 g/L h), and produced more propionate (6.3 vs. 4.7 g/L) at a higher rate (0.039 ±0.003 vs. 0.030±0.003 g/L h) as compared to the WT (Fig. 4a, b). However, there was no significant difference in the propionate yield between the mutant (0.084±0.08 g/g) and the wild type (0.078±0.08 g/g) (see Table 2). To increase propionic acid production, $CaCO_3$ was added to the medium to buffer the pH and neutralize produced organic acids in the fermentation (Fig. 4c, d). Again, the mutant Pf(*ppc*) showed a 50 % higher glycerol consumption rate (0.104±0.008 vs. 0.073±0.005 g/L h) and 40 % increase in propionate productivity (0.073±0.007 vs. 0.054±0.001 g/L h) as compared to WT. Also, Pf(*ppc*) produced 36 % more propionate (13.1 vs. 9.6 g/L for WT). On the other hand, both WT and Pf(*ppc*) gave the same acetate titer of 0.3 g/L, which was much lower than those in glucose fermentation as glycerol is a more reduced carbon source. Succinate concentrations were 1.3 and 1.7 g/L in the WT and mutant, respectively. The propionate yield from glycerol was 0.83±0.02 g/g for both the mutant and WT, which was ~60 % higher than those from glucose. Although there was no significant difference in the propionate yield from glycerol between the WT and mutant, the mutant gave a significantly higher *P*/*A* ratio (36.8 vs. 29.3 g/g) in the fermentation.

Batch fermentation in bioreactor with pH controlled at 6.5

When Pf(*ppc*) and WT were grown on glucose in a bioreactor controlled at pH 6.5, higher glucose consumption rates (0.23 and 0.17 g/L h, respectively), propionic acid production rates

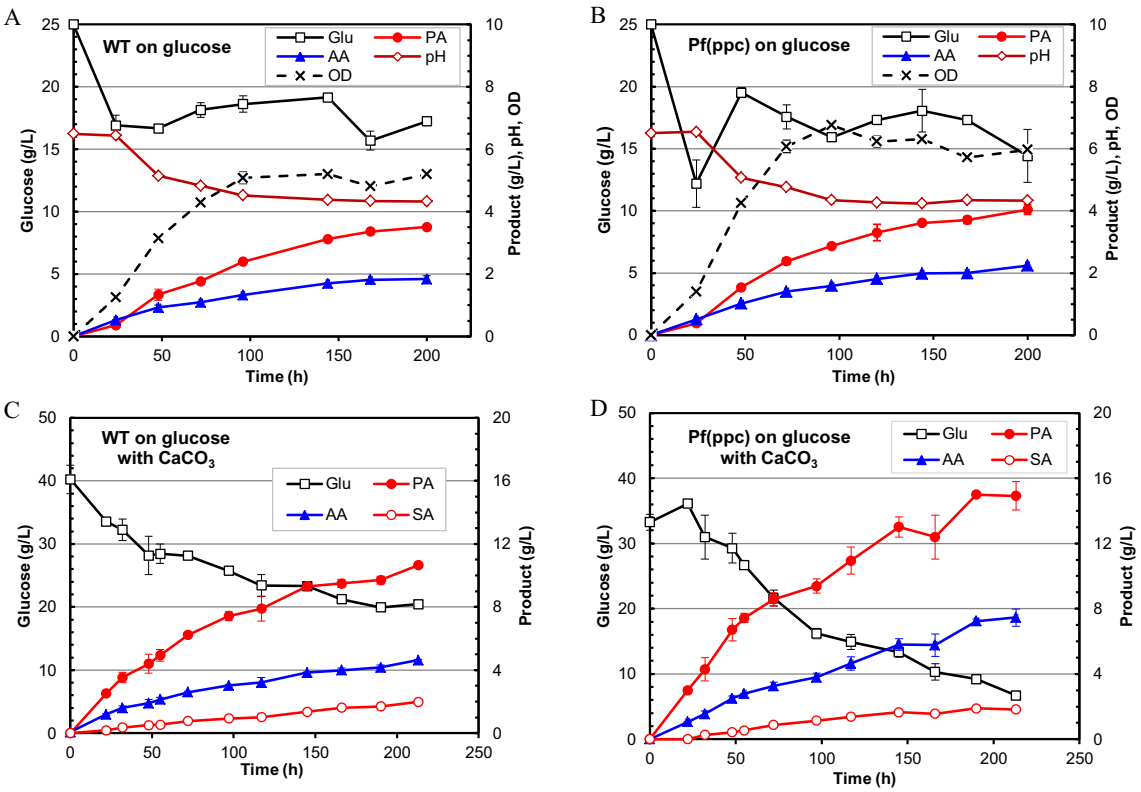

Fig. 3 Batch fermentation kinetics with glucose in serum bottles. **a** WT without $CaCO_3$. **b** Pf(*ppc*) without $CaCO_3$. **c** WT with $CaCO_3$. **d** Pf(*ppc*) with $CaCO_3$. Succinic acid was not measured in **a** and **b**. In **c** and **d** with $CaCO_3$, OD was not measured while the pH dropped from the initial ~6.5 to ~5.0 in ~12 h and remained at ~5.0. *Glu* glucose, *PA* propionic acid, *AA* acetic acid, *SA* succinic acid

(0.10 and 0.09 g/L h, respectively), and final propionic acid titers (up to 19.5 g/L) were obtained (Fig. 5a, b) as compared to fermentations at~pH 5.0 in serum bottles. In general, the mutant performed similarly to the WT with regard to propionate production from glucose over three independent batches at pH 6.5. However, when glycerol was used as the carbon source, Pf(*ppc*) had a significantly higher specific growth rate (0.060±0.003 vs. 0.052±0.004/h) and produced more propionate (13.2 vs. ~9 g/L) in 240 h (Fig. 5c, d) with a 68 % higher propionate productivity (0.064±0.006 vs. 0.038±0.009 g/L h) and 173 % higher glycerol consumption rate (0.120±0.014 vs. 0.044±0.005 g/L h) as compared to the wild type (see Table 2). It is clear that the mutant Pf(*ppc*) consumed more and grew faster on glycerol and produced propionate at a higher rate than did the WT. Both *P*/*A* and *P*/*S* ratios were also higher for the mutant, although the propionate yield from glycerol was the same (~0.78 g/g) for both the mutant and WT.

Effects of CO_2 partial pressure

The expression of *ppc* should facilitate CO_2 fixation and the direct conversion of PEP to OAA, thus shifting the carbon flux towards the biosynthesis of propionic acid, instead of acetic acid. To manifest the effect of CO_2 fixation on propionic acid fermentation, batch fermentations of glucose were studied in serum bottles tightly sealed to allow gas pressure buildup to ~2 atm. At the higher CO_2 partial pressure, CO_2 fixation should occur faster and its effect would be more pronounced. For comparison, fermentations were also carried out in serum bottles each installed with a one-way check valve to release the produced gas and maintain the bottles at the ambient pressure. Figure 6 compares the fermentation results from the mutant Pf(*ppc*) and wild type under pressurized and nonpressurized conditions. Compared to the wild type, the mutant gave a faster fermentation with a higher final propionic acid titer (Fig. 6a) and productivity (Fig. 6b), which was consistent with the results shown in Fig. 4. It was also found that without CO_2 pressurization, the mutant had similar *P*/*A* ratio (Fig. 6c) and propionic acid yield (Fig. 6d) to those of the WT, which was also consistent with the results shown in Table 2. However, under pressurized conditions, the mutant produced significantly more propionic acid and less acetic acid from glucose, resulting in a ~33 % increase in the *P*/*A* ratio (3.28 vs. 2.47) and 10.4 % increase in the propionic acid yield (0.51 vs. 0.46 g/g). In contrast, increasing the CO_2 partial pressure in the bottles did not significantly affect the WT fermentation kinetics. The lack of CO_2

Table 2 Comaprison of fermentation kinetics by the mutant Pf(*ppc*) and wild type with glucose and glycerol as carbon source under various pH conditions

	No $CaCO_3$ (no pH control)		With $CaCO_3$ (~pH 5)		Bioreactor (pH 6.5)	
	WT	Mutant	WT	Mutant	WT	Mutant
Glucose						
μ (h^{-1})	0.095±0.001	0.102±0.001*	NA	NA	0.076±0.002	0.078±0.013
Acetate (g/L)	1.8±0.1	2.2±0.1	4.6±0.1	7.5±0.5*	2.9±0.3	3.9±0.9
Succinate (g/L)	NA	NA	2.0±0.0	1.8±0.1	3.6±0.1	5.4±1.2
Propionate (g/L)	3.5±0.1	4.0±0.2*	10.7±0.1	14.9±0.9*	18.9±0.1	19.5±0.2
Total acids (g/L)	5.4±0.1	6.3±0.2*	17.6±0.1	24.5±1.4*	25.7±0.2	27.8±2.1
P/A (g/g)	2.0±0.2	2.0±0.2	2.5±0.1	2.5±0.2	6.7±0.7	5.1±1.0
P/S (g/g)	NA	NA	4.7±0.1	6.9±0.4*	5.3±0.2	3.6±1.1
PA yield (g/g)	0.52±0.07	0.53±0.04	0.51±0.06	0.49±0.08	0.48±0.06	0.51±0.07
Total acid yield (g/g)	0.80±0.10	0.82±0.07	0.82±0.09	0.74±0.15	0.67±0.02	0.68±0.06
PA productivity (g/L h)	0.021±0.001	0.029±0.002*	0.046±0.001	0.066±0.006*	0.093±0.014	0.103±0.025
Total acid productivity (g/L h)	0.031±0.001	0.036±0.004*	0.074±0.001	0.109±0.01*	0.118±0.012	0.155±0.034
Glucose consumption rate (g/L h)	0.040±0.001	0.046±0.003*	0.090±0.020	0.142±0.013*	0.167±0.005	0.227±0.042*
Glycerol						
μ (h^{-1})	0.078±0.003	0.089±0.002*	NA	NA	0.052±0.004	0.060±0.003*
Acetate (g/L)	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.5±0.0	0.3±0.1
Succinate (g/L)	0.3±0.0	0.3±0.0	1.3±0.0	1.7±0.1*	2.2±0.3	1.5±0.5
Propionate (g/L)	4.7±0.3	6.3±0.4*	9.6±0.1	13.1±1.1*	12.8±2.0	13.2±2.2
Total acids (g/L)	5.7±0.3	7.4±0.4*	11.4±0.1	15.4±1.1*	15.5±2.3	15.5±2.8
P/A (g/g)	13.0±0.2	16.7±1.9*	29.3±0.3	36.8±2.5*	21.2±4.7	37.5±4.4*
P/S (g/g)	10.4±1.2	11.3±2.7	8.3±0.3	8.2±0.8	6.0±0.4	9.8±2.3
PA yield (g/g)	0.78±0.08	0.86±0.08	0.83±0.01	0.82±0.02	0.78±0.02	0.78±0.02
Total acid yield (g/g)	0.85±0.09	0.94±0.08	0.98±0.06	0.89±0.02	0.89±0.30	0.96±0.15
PA productivity (g/L h)	0.030±0.003	0.039±0.003*	0.054±0.000	0.073±0.007*	0.038±0.009	0.064±0.006*
Total acid productivity (g/L h)	0.033±0.003	0.042±0.003*	0.071±0.000	0.090±0.009*	0.044±0.010	0.071±0.009*
Glycerol consumption (g/L h)	0.038±0.002	0.053±0.004*	0.073±0.005	0.104±0.008*	0.044±0.005	0.120±0.014*

WT wild type, *NA* not available

*$p<0.05$, statistically significantly higher

effect on the WT is as expected since WT does not have PPC or PYC needed to fix CO_2 according to the published whole genome sequences (Falentin et al. 2010; Meurice et al. 2004).

Discussion

The genetic manipulation of propionibacteria is relatively difficult because of the limited availability of cloning vectors and low transformation efficiency (Cheong et al. 2008; Genevieve 1988; Jore et al. 2001; Luijk et al. 2002). To date, a few expression vectors such as pKHEM01 and pKHEM04 with promoters p138 and p4, respectively (Kiatpapan et al. 2000; Kiatpapan and Murooka 2001, 2002), and two promoterless reporter vectors (pTD210 and pCVE1) (Faye et al. 2008; Piao et al. 2004a) have been developed to overexpress genes in propionibacteria. However, most of the prior studies focused on vitamin B_{12} production by *P. freudenreichii* (Piao et al. 2004b). To date, only a few studies were on the metabolic engineering of propionibacteria for enhanced propionic acid production (Ammar et al. 2013; Suwannakham et al. 2006; Zhuge et al. 2013). In our recent study, *P. freudenreichii* DSM 4902 was engineered to produce *n*-propanol by expressing an exogenous aldehyde/alcohol dehydrogenase (*adhE*), which also increased propionate productivity especially when glycerol was the carbon source (Ammar et al. 2013). Zhuge et al. (2013) cloned and expressed the glycerol dehydrogenase gene (*gldA*) from *Klebsiella pneumoniae* in *Propionibacterium jensenii* ATCC 4868, and the mutant showed about 30 % increase in propionate production from glycerol compared to the wild type strain.

In the present study, we aimed at increasing the carbon flux towards OAA in the dicarboxylic acid pathway to enhance

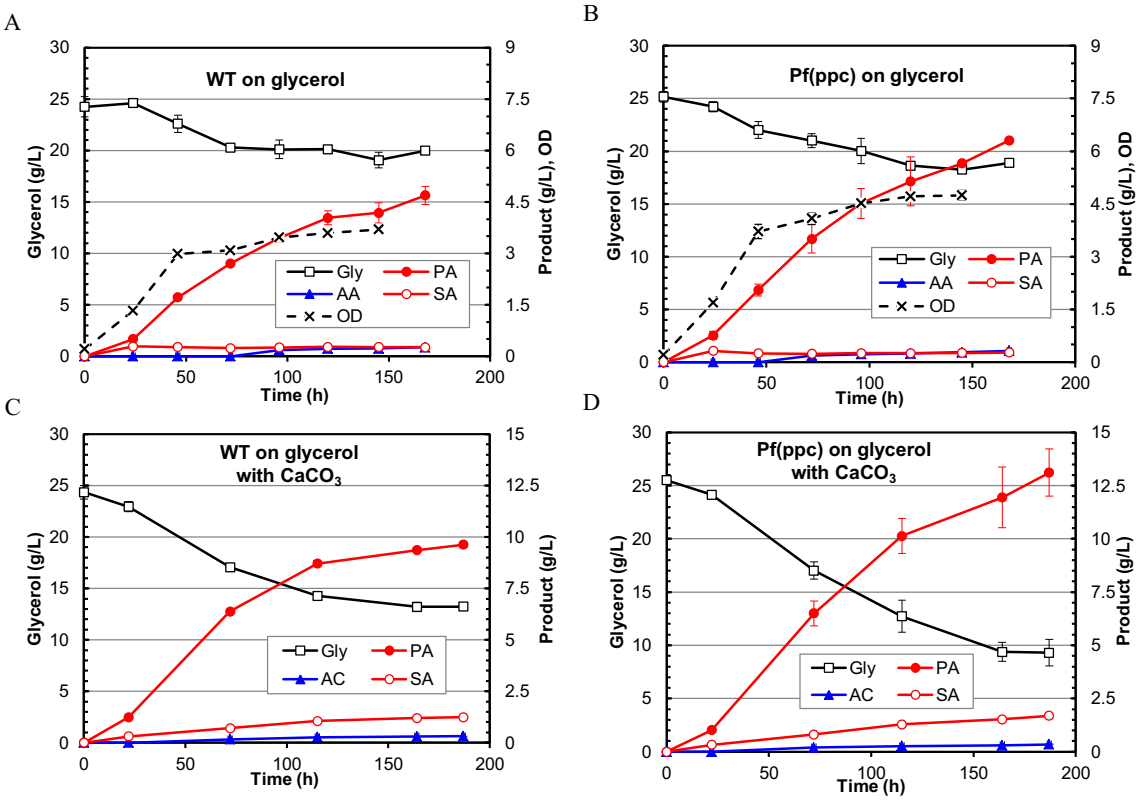

Fig. 4 Batch fermentation kinetics with glycerol in serum bottles. **a** WT without $CaCO_3$. **b** Pf(*ppc*) without $CaCO_3$. **c** WT with $CaCO_3$. **d** Pf(*ppc*) with $CaCO_3$. Without $CaCO_3$ (**a**, **b**), the pH dropped from the initial ~6.5 to ~5.0 in ~50 h and continued to gradually decrease to ~4.5; with $CaCO_3$ (**c**, **d**) the pH dropped from the initial ~6.5 to ~5.0 in ~24 h and remained at ~5.0. *Glu* glucose, *PA* propionic acid, *AA* acetic acid, *SA* succinic acid

propionic acid biosynthesis by overexpressing *ppc* in *P. freudenreichii* DSM 20271. We chose to overexpress *ppc* instead of *pyc* based on the proven outcomes from similar studies on other microbes (Millard et al. 1996; Song et al. 2013; Zhang et al. 2012). In theory, expressing PPC for the direct conversion of PEP to OAA to bypass pyruvate should increase carbon flux towards propionate and decrease acetate production. To manifest this effect, we first studied the fermentation kinetics in serum bottles (~pH 5) and bioreactor (pH 6.5) with either glucose or glycerol as carbon source under the ambient pressure. However, the *ppc*-expressing mutant did not show any significant increase in propionate yield or decrease in acetate production. This was probably because the biosynthesis of propionyl-CoA, the precursor for propionic acid, is associated with the conversion of pyruvate to OAA. Thus, bypassing the reaction from pyruvate to OAA could have also prevented the production of propionyl-CoA and propionic acid. Therefore, no significant effect on the carbon flux partitioning between acetate and propionate biosynthesis was observed. It should be noted that the coproduction of acetate and propionate from glucose is necessary for balancing NADH/NAD^+, and therefore, increasing the carbon flux to OAA without increasing NADH may not be sufficient to increase propionic acid biosynthesis. Nevertheless, the mutant had a significantly higher specific growth rate and fermentation productivity, which could be partially attributed to the higher cell density reached in the fermentation (see Figs. 3, 4, and 5). However, the higher cell density itself cannot fully account for the increased substrate consumption rate and productivity, suggesting that the mutant had a higher specific productivity. Thus, the *ppc*-expressing mutant could be used to effectively increase propionic acid production rate.

The higher propionate production rate and titer obtained with the mutant in serum-bottle fermentations, where the pH was maintained at ~5 or lower (see Figs. 3 and 4), also suggested that the *ppc*-expressing mutant had better tolerance to propionic acid, which is a strong inhibitor to cells. The increased propionic acid production or tolerance could be attributed to the increased succinate production which would increase the reaction converting propionyl-CoA to propionic acid catalyzed by propionyl-CoA, succinate CoA transferase. This finding was consistent with the results that the mutant grew to a higher final cell density in the presence of 5–10 g/L propionic acid (see Fig. 2). However, the mutant's propionic acid tolerance advantage diminished when the propionic acid concentration increased to 20 g/L or became less noticeable in the bioreactor fermentation with pH controlled at 6.5 (see Fig. 5). The latter is understandable as only the undissociated propionic

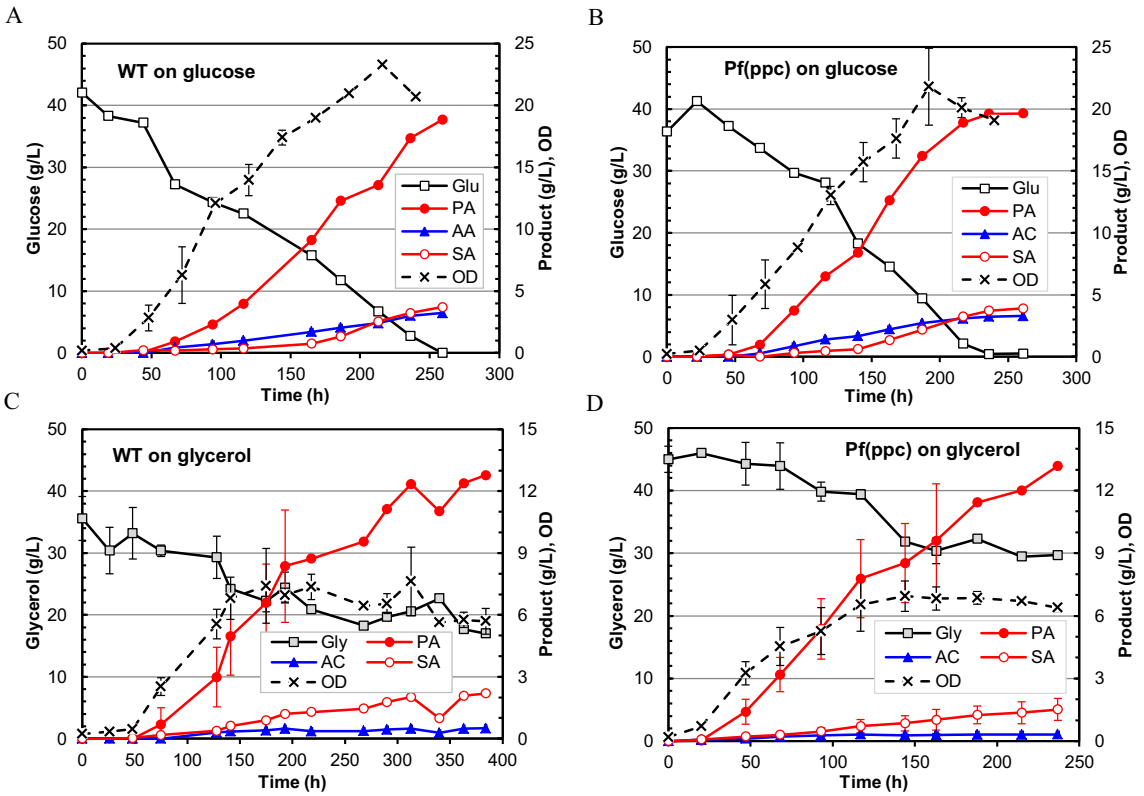

Fig. 5 Batch fermentation kinetics in bioreactor with pH controlled at ~6.5. **a** WT on glucose. **b** Pf(*ppc*) on glucose. **c** WT on glycerol. **d** Pf(*ppc*) on glycerol. *Glu* glucose, *Gly* glycerol, *PA* propionic acid, *AA* acetic acid, *SA* succinic acid

acid is toxic to cells while at pH 6.5, most of the propionic acid is present as salt or in the dissociated form, which is not as toxic to cells (Gu et al. 1998; Hsu and Yang 1991).

It is noteworthy that the effects of *ppc* expression were more profound in fermentations with glycerol than with glucose as the carbon source. Glucose being a more oxidized carbon source, compared to glycerol, stimulates acetate formation to provide additional NADH to support propionate biosynthesis and maintain the intracellular NADH balance (Wang and Yang 2013). Consequently, the effects of PPC on flux distribution and fermentation kinetics (i.e., substrate consumption rate, cell growth rate, and propionate production rate) were not as profound with glucose compared to glycerol. On the other hand, more propionate can be produced from glycerol than from glucose as glycerol being the more reduced substrate would favor the more reduced product, propionic acid (Coral et al. 2008; Himmi et al. 2000; Liu et al. 2011; Zhang and Yang 2009b). Compared to the WT, the *ppc*-expressing mutant consistently gave a higher *P*/*A* ratio in glycerol fermentations under all conditions studied (see Table 2), confirming our hypothesis that expressing *ppc* would shift some carbon flux towards propionate instead of acetate. However, the propionate yield from glycerol was not increased, probably because more carbon source was used for cell growth, resulting in more biomass formation and faster glycerol consumption and propionate production in the fermentation. Overexpressing *ppc* did not significantly change the *P*/*S* ratio since both propionate and succinate are downstream of OAA in the dicarboxylic acid pathway.

The effects of *ppc* expression on propionic acid production were further manifested in glucose fermentation under increased CO_2 pressure, which should increase CO_2 fixation and thus produce a more pronounced effect on carbon flux redistribution. Indeed, the elevated CO_2 partial pressure in tightly sealed bottles significantly increased propionate production from glucose, resulting in a 10.4 % increase in the propionate yield and ~33 % increase in the *P*/*A* ratio (see Fig. 6), which confirmed that there were significant amounts of OAA formed directly from PEP because of the fixation of CO_2 in the *ppc*-expressing mutant. The increased propionic acid production by the mutant could be attributed to the increased CO_2 fixation and succinic acid biosynthesis. Kinetically, increasing the concentration of succinic acid in cells should also increase the reaction catalyzed by propionyl-CoA, succinate CoA transferase, which is believed to be a rate-limiting step in the Wood-Werkman cycle, and thus can increase propionic acid biosynthesis. This in turn should also increase the reaction from pyruvate to OAA and thus alter the flux distribution at the pyruvate branch point to favor propionic acid biosynthesis. It should be noted that some propionibacteria such as *P. acidipropionici* has MMD that can decarboxylate methylmalonyl-CoA to propionyl-CoA and CO_2. However,

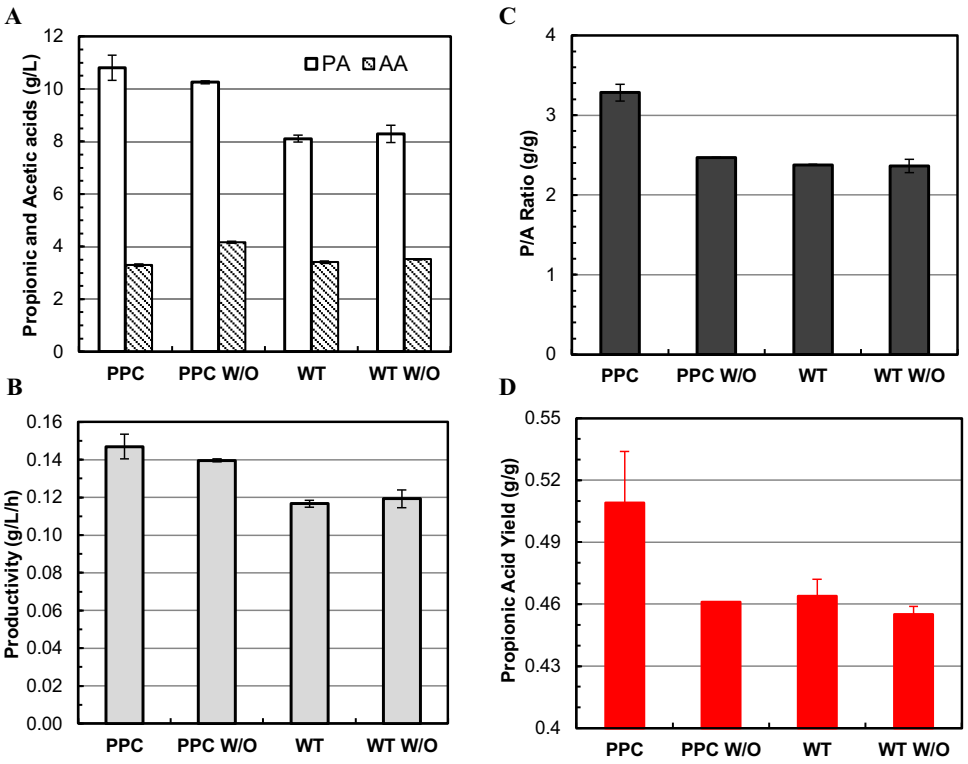

Fig. 6 Effects of CO_2 pressure on propionic acid production from glucose in serum bottles with the mutant (PPC) and wild type (WT). *W/O* without CO_2 pressurization. **a** Propionic acid and acetic acid titers. **b** Propionic acid productivity. **c** Propioinic acid/acetic acid (*P*/*A*) ratio. **d** Propionic acid yield. *PA* propionic acid, *AA* acetic acid

this reaction cannot explain the increased propionic acid biosynthesis since there is no MMD gene found in the genome of *P. freudenreichii*.

In general, the result supports our hypothesis that the expression of *ppc* should facilitate CO_2 fixation and the direct conversion of PEP to OAA, bypassing pyruvate and shifting the carbon flux towards the biosynthesis of propionic acid, instead of acetic acid. The data suggests that the observed effects on the mutant cannot be attributed to the increased total pressure in the bottle as no effect was observed with the wild type under the same condition. One would have expected to see the same effect for both the mutant and wild type if the increased propionic acid production were solely caused by the increased total pressure instead of CO_2 partial pressure. It is also noted that increasing the total pressure might also increase propionic acid production by the mutant because of increased CO_2 solubility (and availability) at elevated pressure. However, this will not change the conclusion that increasing CO_2 partial pressure (or solubility) increased the carbon flux through OAA and resulted in higher propionic acid production.

Nevertheless, at the ambient pressure or without CO_2 pressurization, the increased flux through OAA in the mutant did not significantly increase propionate yield. It is possible that the increased CO_2 fixation via the PPC-catalyzed reaction also allowed more CO_2 to be produced in the acetate biosynthesis pathway, which also produced more ATP to support cell maintenance and growth. Without PPC or the ability to fix CO_2 generated in the acetate pathway, the produced CO_2 or carbonic acid inside the cells could inhibit cell growth and fermentation, which may explain why the mutant grew faster and consumed more glycerol in the fermentation as compared to the wild type. Furthermore, the significantly increased propionic acid yield from glucose under the elevated CO_2 pressure also suggested that increasing CO_2 pressure not only may increase CO_2 fixation and propionic acid production but also could inhibit acetate and CO_2 production; however, this remains to be verified. In summary, the ability of the mutant to fix CO_2 increased flux from PEP to OAA, cell growth, and cell specific activity, resulting in increased substrate consumption and propionate production in the fermentation.

In conclusion, expressing *ppc* in *P. freudenreichii* DSM 20271 can improve propionate production. With glycerol, the more reduced substrate, as the carbon source, the limitation of required cell maintenance energy and reducing equivalents is mitigated, and the mutant expressing *ppc* gene becomes distinctly more productive and gives a higher *P*/*A* ratio compared to the wild type. To the best of our knowledge, this is the first report on metabolic engineering of propionibacteria for propionic acid production by upregulating the propionate branch of the dicarboxylic acid pathway using exogenous *ppc* gene.

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