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Development of a *Propionibacterium-Escherichia coli* Shuttle Vector for Metabolic Engineering of *Propionibacterium jensenii*, an Efficient Producer of Propionic Acid

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Propionic acid (PA) is an important chemical building block and is widely applied for organic synthesis, food, feedstuff, and pharmaceuticals. To date, the strains that can efficiently produce PA have included *Propionibacterium thoenii*, *P. freudenreichii*, and *P. acidipropionici*. In this report, we show that *P. jensenii* ATCC 4868 is also able to produce PA in much higher yields than the previously reported strains. To further improve the production capacity, a *P. jensenii-Escherichia coli* shuttle vector was developed for the metabolic engineering of *P. jensenii*. Specifically, a 6.9-kb endogenous plasmid, pZGX01, was isolated from *P. acidipropionici* ATCC 4875 and sequenced. Since the sequencing analysis indicated that pZGX01 could encode 11 proteins, the transcriptional levels of the corresponding genes were also investigated. Then, a *P. jensenii-Escherichia coli* shuttle vector was constructed using the pZGX01 plasmid, the *E. coli* pUC18 plasmid, and a chloramphenicol resistance gene. Interestingly, not only could the developed shuttle vector be transformed into *P. jensenii* ATCC 4868 and 4870, but it also could be transformed into *freudenreichii* ATCC 6207 subspecies of *P. freudenreichii*. Finally, the glycerol dehydrogenase gene (*gldA*) from *Klebsiella pneumoniae* was expressed in *P. jensenii* ATCC 4868 with the constructed shuttle vector. In a 3-liter batch culture, the PA production by the engineered *P. jensenii* ATCC 4868 strain reached 28.23 ± 1.0 g/liter, which was 26.07% higher than that produced by the wild-type strain (22.06 ± 1.2 g/liter). This result indicated that the constructed vector can be used a useful tool for metabolic engineering of *P. jensenii*.

Propionic acid (PA) is a valuable C3 platform chemical (three-carbon compound that can be used to synthesize a series of products with high added value) that is widely used as a preservative in animal feed and human foods. It is also an important chemical intermediate for the synthesis of cellulose fibers, herbicides, perfumes, and pharmaceuticals (1, 2). According to the U.S. Department of Energy, PA is among the top 30 candidate platform chemicals used for a variety of applications (3). Industrially, PA is produced from petrochemical raw materials (e.g., ethylene and carbon monoxide) via oxo-synthesis (4). Due to exhaustion of petroleum resources and the serious environmental pollution caused by the utilization of fossil resources, the production of PA by microbial fermentation from renewable resources has attracted increasing attention (5). To date, the species used for PA production have included *Propionibacterium freudenreichii* (6), *P. acidipropionici* (5, 7–14), and *P. thoenii* (15). Almost all the reports on this topic mainly focused on the optimization of the process (6–8, 11, 14, 16) and helped to significantly improve the PA production levels. However, the biotechnological production of PA cannot be achieved at industrial scale due to the weak economic competiveness compared with the conventional chemical synthesis route. Therefore, it seems that there is not much room for further enhancement of PA production by process optimization, and thus, it is necessary to develop metabolic engineering strategies aiming at further enhancing PA production.

Several vectors have been isolated from dairy propionibacteria such as *P. freudenreichii* and *P. acidipropionici* (17–20). The reported *Propionibacterium* plasmids include pLME108 from *P. freudenreichii* DF2, pLME106 from *P. jensenii* DF1 (21), pRGO1 from *P. acidipropionici* E214 (19), and p545 from *P. freudenreichii* LMG 16545 (18). To date, four *Propionibacterium-Escherichia coli* shuttle vectors have been developed by using these plasmids (18, 19, 21, 22). Kiatpapan et al. (19) developed the *Propionibacterium-E. coli* shuttle vector pPK705 by using plasmids pRG01 and pUC18 and the hygromycin B resistance gene. Jore et al. (18) established a reproducible transformation approach for *P. freudenreichii* with plasmid p545 from *P. freudenreichii* itself and tested the erythromycin resistance gene and the chloramphenicol resistance gene as the selection marker. The successful transformation of *P. freudenreichii* was also achieved with two different vectors based on the plasmids pLME106 and pLME108 (21). Brede et al. (22) constructed the *Propionibacterium-E. coli* shuttle vector pSL104 by using plasmid pLME108 and a propionicin F bacteriocin immunity gene. Unfortunately, these reported vectors can only be transformed into *P. freudenreichii* (17–19, 21) and *P. acnes* (23), and thus, the genetic manipulation has been limited to these two organisms. For example, the *hemA*, *hemB*, and *choA* genes were overexpressed for the production of vitamin B_{12} in *P.*

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TABLE 1 Plasmids and strains used in this study[a]

Plasmid or strain	Relevant characteristic(s)	Source
Plasmids		
pUC18	*E. coli* cloning vector; Ap^r	TaKaRa
pZGX01	Wild plasmid in *P. acidipropionici* ATCC 4875	This study
pZGX04	*E. coli*-PAB shuttle vector Ap^r in *E. coli* and Cm^r in PAB; derived from pUC18, pZGX02, and *cml*/*cmx* PCR product	This study
pZGX04-*gldA*	*gldA* expression vector	This study
Strains		
E. coli JM109	Clone host	TaKaRa
E. coli JM110	*dam* and *dcm* mutant conferring no methylation of DNA	Stratagene
K. pneumonia subsp. *pneumoniae* ATCC 12657	Carries *gldA*	ATCC
P. acidipropionici ATCC 4875	Carries plasmid pZGX01	ATCC
P. acidipropionici ATCC 4965	No native plasmid; electrotransformation	ATCC
P. acidipropionici ATCC 25562	No native plasmid; electrotransformation	ATCC
P. jensenii ATCC 4868	No native plasmid; electrotransformation; transformable by shuttle vector pZGX04	ATCC
P. jensenii ATCC 4868 (pZGX04-*gldA*)	*P. jensenii* ATCC 4868 carries pZGX04-*gldA*	This study
P. jensenii ATCC 4870	No native plasmid; electrotransformation; transformable by shuttle vector pZGX04	ATCC
P. freudenreichii subsp. *freudenreichii* ATCC 6207	No native plasmid; electrotransformation; transformable by shuttle vector pZGX04	ATCC
P. freudenreichii subsp. *shermanii* ATCC 9614	No native plasmid; electrotransformation	ATCC
P. thoenii ATCC 4872	No native plasmid; electrotransformation	ATCC
P. thoenii ATCC 4874	No native plasmid; electrotransformation	ATCC

[a] Ap^r, ampicillin selection; Cm^r, chloramphenicol selection; *cml*/*cmx*, complete *cml*(A) and *cmx*(A) genes; ATCC, American Type Culture Collection, Manassas, VA.

freudenreichii (24, 25). The only report about the metabolic engineering of *Propionibacterium* for the enhancement of PA production focused on the deletion of the byproduct acetic acid-encoding gene in *P. acidipropionici* (12). The slow development of genetic engineering of *Propionibacterium* is mainly due to the lack of detailed genome information and transformable plasmids (26), although several species, including *P. acidipropionici*, *P. propionicum*, and *P. freudenreinchii*, have already been completely sequenced.

Although *P. jensenii* belongs to the dairy propionibacteria, most of the studies reporting on this organism were focused on the production of bacteriocin, antimicrobial peptides, and the polyene pigment granadaene (27–30). In this work, we reported that *P. jensenii* ATCC 4868 is capable of efficiently producing PA in large amounts. Currently, there is no transformable plasmid for *P. jensenii* to be used for strengthening the PA synthesis capacity of *P. jensenii*. Therefore, we constructed a *P. jensenii-Escherichia coli* shuttle vector as an engineering tool for gene expression in *P. jensenii*. Moreover, the glycerol dehydrogenase-encoding gene (*gldA*) from *Klebsiella pneumoniae* was expressed in *P. jensenii* ATCC 4868, resulting in PA production that was noticeably higher than that of the wild-type strain. This work may contribute to the development of genetic manipulation of *Propionibacterium* strains for highly efficient production of PA.

MATERIALS AND METHODS

Bacterial strains, vectors, and media. *Propionibacterium* strains were purchased from American Type Culture Collection (ATCC). *E. coli* JM109 and pUC18 plasmid were purchased from TaKaRa (Dalian, China). *E. coli* JM110 was purchased from Stratagene (La Jolla, CA). pXZ10145, a plasmid containing a chloramphenicol resistance gene (*cml*/*cmx*), was provided by Z. X. Zheng (Fudan University, Shanghai, China). The bacterial strains and vectors used in this work are shown in Table 1. *E. coli* was cultured at 37°C in Luria-Bertani (LB) medium containing 1% (wt/vol) NaCl, 1% (wt/vol) peptone, and 0.5% (wt/vol) yeast extract supplemented with 100 μg/ml ampicillin. *Propionibacterium* was grown anaerobically at 30°C in sodium lactate broth (SLB) medium containing 1% (wt/vol) sodium lactate, 1% (wt/vol) yeast extract, and 1% (wt/vol) Trypticase soy broth supplemented with 10 μg/ml chloramphenicol.

DNA isolation and manipulation. The plasmids from *Propionibacterium* and *E. coli* were extracted using a MiniBEST plasmid purification kit (TaKaRa, Dalian, China) or a Sangong Biotech large-scale plasmid purification kit (Sangong, Shanghai, China). The genomic DNA of *Propionibacterium* strains was prepared using a UNlQ-10 column bacterial genomic DNA isolation kit (Sangong, Shanghai, China). The *Propionibacterium* cells were treated with 10 mg/ml lysozyme and 100 U/ml mutanolysin for 30 min before plasmid extraction. Restriction enzymes and T4 DNA ligase (TaKaRa, Dalian, China) were used according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). To conduct transcriptional analysis of the isolated plasmids, total RNA was extracted from the cells when their optical density at 600 nm (OD_{600}) reached 0.7 in SLB medium. The RNA was isolated using an RNAisoPlus kit (TaKaRa, Dalian, China), and the cells were suspended in a lysozyme solution (40 mg/ml) for pretreatment. The total RNA was treated with a PrimeScript II first-strand cDNA synthesis kit (TaKaRa, Dalian, China). The obtained cDNA was subjected to quantitative PCR (qPCR) using the LightCycler 2.0 system (Roche, Basel, Switzerland) and SYBR *Premix Ex Taq* (TaKaRa, Dalian, China). Primers were designed by using Primer Premier software (version 5.00; PREMIER Biosoft International, Palo Alto, CA) and are listed in Table S1 in the supplemental material.

Transformation of *Propionibacterium* strains. The transformation optimization was conducted based on several previous reports (17, 19, 21). *Propionibacterium* strains were cultured up to the stationary-growth phase, and then the cultures were diluted 50 times with SLB medium. After incubation for 20 h, the cells in the exponential-growth phase (OD_{600} = 0.7) were kept on ice for 30 min. After centrifugation at 5,000 × *g* for 4 min, the cells were washed twice with ice-cold sucrose (0.5 M). Finally, the cells were resuspended in a 0.01 volume of ice-cold electroporation buffer (0.5 M sucrose, 1 mM potassium acetate, pH 5.5). The transformation was conducted by electroporation using a Gene Pulser apparatus (Bio-Rad, Hercules, CA). Eighty microliters of the cell suspension was

mixed with 1.5 μg of plasmid in an ice-cold electroporation cuvette and kept on ice for 30 min. Then, an electric pulse was delivered at a field strength of 12.5 kV/cm with a resistance of 200 Ω. After the pulse, 900 μl of ice-cold SLB medium containing 0.5 M sucrose was added. After 3 h of incubation at 30°C, the cells were spread on SLB agar plates containing 10 μg/ml chloramphenicol. The transformants could be observed after incubation at 30°C for 7 to 10 days under anaerobic conditions.

DNA sequencing. To determine the complete nucleotide sequence of pZGX01, the plasmid was linearized by BamHI and subcloned in pUC18 for sequencing (fold coverage, 30×), which was performed by TaKaRa (Dalian, China). The sequencing data were assembled and analyzed by using Vector NTI (version 11; Invitrogen, New York, NY), GLIMMER (version 3.2; Center for Bioinformatics and Computational Biology, University of Maryland [http://www.cbcb.umd.edu]), Promoter 2.0 (http://www.cbs.dtu.dk/services/Promoter), GeneMarkS (version 4.7; Georgia Institute of Technology, Atlanta, GA [http://opal.biology.gatech.edu/GeneMark/genemarks.cgi]), and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) software.

Fermentation conditions. The inoculums were prepared in 250-ml anaerobic jars containing 200 ml of sterile culture medium supplemented with 10 g/liter yeast extract, 5 g/liter Trypticase soy broth, 2.5 g/liter K_2HPO_4, and 1.5 g/liter KH_2PO_4. The jars were sealed with butyl rubber caps, and the cultures were incubated at 30°C for 48 h. Batch fermentations were performed under anaerobic conditions with a nitrogen flux in a 3-liter BIOFLO 115 Bioreactor (Eppendorf, Hamburg, Germany) containing 2 liters of culture medium supplemented with 30 g/liter glycerol. The medium was autoclaved at 121°C for 21 min and then purged with sterile nitrogen gas to remove any traces of oxygen before the inoculation. The temperature and agitation speed were maintained at 30°C and 200 rpm, respectively, and the pH was controlled at 7.0 via automatic addition of $Ca(OH)_2$ (10% [wt/vol]). About 200 ml of seed culture was used as the inoculum.

Analytical methods. Fifteen-milliliter aliquots were drawn from the fermentor every 12 h, and 3 ml was used to determine the OD_{600} with a UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan) after an appropriate dilution. Dry cell weight (DCW) was calculated from the OD_{600} according to the following equation: DCW (g/liter) = 0.23 × OD_{600}.

Fifteen-milliliter aliquots were used for the quantification of acetic acid, succinic acid, and PA concentrations. The samples were centrifuged at 7,000 × *g* for 10 min, and the supernatant was filtered with a 0.22-μm-pore-size-filter membrane (diameter, 25 mm). The sample was analyzed by the use of an Agilent 1200 high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA) equipped with a Zorbax SB-Aq column (Agilent, Santa Clara, CA) (250 by 4.6 mm), a standard G 1329A autosampler (Agilent, Santa Clara, CA), and a G13158 diode array detector (DAD) (Agilent, Santa Clara, CA). Na_2HPO_4 (0.138 mol/liter) and acetonitrile (1% [vol/vol]), the pH of which was adjusted to 2.0 with phosphoric acid, were used as the mobile phase at a flow rate of 1.0 ml/min. The detection wavelength was 210 nm, and the column temperature was maintained at 35°C. The concentration of the products was calculated by comparing the peak areas with that of the internal standard.

Three-milliliter aliquots were used for analyzing glycerol dehydrogenase activity. The samples were centrifuged at 10,000 × *g* for 5 min, and then the cell pellets were washed with 0.5 ml of K_2HPO_4-KH_2PO_4 buffer (50 mM, pH 7.0) and resuspended in the same buffer. The cell suspension was sonicated to break up the cell walls and centrifuged at 10,000 × *g* and 4°C for 15 min to remove cell debris. The reaction mixture (1 ml) contained 30 mM ammonium sulfate, 0.2 M glycerol, and 1.2 mM NAD (adjusted to pH 7.0 with 1 M NaOH), and the supernatant of the lysate in 0.1 M potassium carbonate buffer solution (pH 9.8). The absorption intensity was measured by a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan) at 340 nm and 30°C. One unit of activity was defined as the amount of enzyme required to reduce 1 mmol of substrate per minute under the specified conditions.

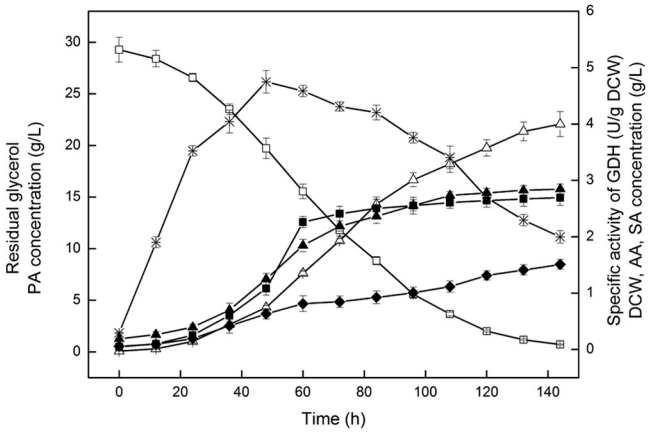

FIG 1 Batch fermentation kinetics of propionic acid production from glycerol with *P. jensenii* ATCC 4868 at pH 7.0 and 30°C for 144 h. The initial glycerol concentration was 30 g/liter; samples were taken from the fermentor every 12 h. △, propionic acid (PA); □, residual glycerol; ▲, dry cell weight (DCW); ■, acetic acid (AA); ◆, succinic acid (SA); *, specific activity of GDH.

Stability of the vector in *Propionibacterium*. The transformants were grown in SLB medium supplemented with chloramphenicol (10 μg/ml) for 3 days. The culture was diluted by 50 times with fresh SLB medium and cultured at 30°C for 2 days. To determine the plasmid stability, at least 50 colonies from each tested transformant were transferred to SLB agar plates with or without chloramphenicol after 30 serial transfers. The growth of these colonies was monitored after 7 days of incubation. The plasmids in these colonies were analyzed by colony PCR and sequenced. The percentage of stability was determined as follows: number of colonies grown on the selective medium/number of colonies grown on the nonselective medium × 100%.

Statistical analysis. All the experiments were independently performed at least three times, and the results were expressed as means ± standard deviations (SD). Statistical analyses were performed using Student's *t* test. *P* values of less than 0.05 were considered statistically significant.

Nucleotide sequence accession number. The obtained nucleotide sequence data have been deposited in the GenBank database under accession number JQ728013.

RESULTS AND DISCUSSION

Comparison of PA production in batch culture by different *Propionibacterium* species. The reported species used for PA production include *P. freudenreichii* (6), *P. acidipropionici* (5, 7–14), and *P. thoenii* (15). Among those, *P. acidipropionici* is the main producer of PA, with a maximal yield of 97 g/liter from a 3-month fed-batch culture of a PA-tolerant *P. acidipropionici* mutant in a fibrous bed bioreactor (31). Figure 1 shows the time profiles of PA production by batch culture of *P. jensenii* ATCC 4868. The highest PA production reached 22.06 ± 1.2 g/liter after about 145 h, with a yield of 0.152 g · liter^{-1} · h^{-1}. Comparisons of levels of PA production in batch culture by different *Propionibacterium* strains reported in the literature are listed in Table 2. It can be seen that compared to *P. acidipropionici* and *P. thoenii*, *P. jensenii* ATCC 4868 displayed a competitive PA titer and $Y_{PA/substrate}$ (the mass ratio of PA to substrate), indicating that *P. jensenii* ATCC 4868 had a great potential for PA production. To further strengthen the PA synthesis capability of *P. jensenii* ATCC 4868, it was necessary to develop an engineering tool for the genetic manipulation of *P. jensenii* ATCC 4868.

TABLE 2 Production of PA in batch fermentation with different strains[a]

Strain	Substrate	PA (g/liter)	$Y_{P/A}$ (g/g)	$Y_{P/S}$ (g/g)	$Y_{PA/substrate}$ (g/g)	Productivity (g liter^{-1} h^{-1})	Source or reference
P. thoenii	Glycerol	5			0.25	0.03	15
P. acidipropionici ATCC 25562	Glycerol	12.7	5.73	16.53	0.635	0.42	10
P. acidipropionici ATCC 4875	Lactose	22	2.82	2.12	0.44	0.25	9
P. acidipropionici ATCC 4875 adapted mutant	Xylose	19.5	3.45	5.45		0.33	12
P. acidipropionici ATCC 4965	Lactate	15.06	2.69		0.502		7
P. acidipropionici ATCC 4875 (ACK-Tet)	Glycerol	19.3		13.4	0.55	0.026	13
P. acidipropionici CGMCC 1.2230	Glycerol	15.72	13.1	6.81	0.79	0.13	16
P. acidipropionici CGMCC 1.2225	Glycerol/glucose	21.9	25.17	18.4	0.572	0.152	11
P. acidipropionici DSM 4900	Glycerol	19.5	17.85	5.99	0.464	0.34	8
P. acidipropionici ATCC 4875	Arabinose	13.8					5
P. acidipropionici CGMCC 1.2225	Glucose	18.9			0.45	0.13	14
P. freudenreichii CCTCC M207015	Glucose	14.6	4.69	14.44	0.37	0.12	6
P. jensenii ATCC 4868	Glycerol	22.06	8.2	14.61	0.74	0.152	This study
P. jensenii ATCC 4868 (pZGX04-*gldA*)	Glycerol	28.23	9.08	23.52	0.94	0.2	This study

[a] $Y_{P/A}$, the mass ratio of PA to acetic acid; $Y_{P/S}$, the mass ratio of PA to succinic acid.

Plasmid screening and sequencing. Since the previous attempts to transform plasmids originating from other bacteria into *Propionibacterium* failed (19), naturally occurring plasmids in *Propionibacterium* should be used while attempting vector construction. Several plasmids have been screened from dairy *Propionibacterium* (19, 20, 32, 33).

In this work, nine *Propionibacterium* strains, representing all four recognized species of dairy *Propionibacterium*, were used to screen for the presence of endogenous plasmids (Table 1). Only one plasmid, named pZGX01, could be isolated from *P. acidipropionici* ATCC 4875, while no plasmids were found in the other eight strains tested. The complete nucleotide sequence of pZGX01 was determined. pZGX01 plasmid is 6,868 bp long and, in agreement with other *Propionibacterium* sequences, displays a GC content of 65%. The sequence analysis of pZGX01 revealed that it carries 11 open reading frames (ORFs, designated *orf1* to *orf11*), where *orf1*, *orf4*, and *orf5* have opposite orientations with respect to the others. The GC content of most of these ORFs is above 60%, except for *orf9* (51.3%) and *orf10* (44%).

Prediction of ORF function. The features of these 11 ORFs are summarized in Table 3. The proteins related to replication are encoded by *orf6* and *orf7*. All the above-mentioned replication proteins show motifs that are typical of theta-replicating plasmids (34). Propionicin SM1, the first bacteriocin encoded by a *Propionibacterium* plasmid, is related to *orf9* and *orf10* (35). The derived amino acid sequence of *orf10* is believed to originate three transmembrane helices. The gene of *orf10*, encoding a putative protein of unknown function, also has a low GC content (44%). Interestingly, the three predicted transmembrane helices of *orf10* were suggested to be involved in the excretion of propionicin SM1 (21).

According to the literature, four plasmids isolated from *Propionibacterium* have been analyzed (18, 19, 21, 36). Plasmid pPG01 from *P. granulosum* PF283 has three ORFs, which were predicted to be responsible for DNA transfer and plasmid replication (36). Moreover, plasmid pRGO1 from *P. acidipropionici* E214 has six ORFs with GC contents ranging from 61.5 to 73.7% and is predicted to encode two replication proteins (i.e., ORF1 and ORF2) (19). Two replication proteins were also predicted for plasmid p545 from *P. freudenreichii* LMG 16545 (18). Plasmid pLME106 from *P. jensenii* DF1 contains 10 ORFs; among these ORFs, one was predicted to encode a DNA binding protein, one a DNA invertase, and another one propionicin SM1 (21).

Transcriptional analysis of the ORFs. Total RNA was extracted from *P. acidipropionici* ATCC 4875 when the OD_{600} reached 0.7 in SLB medium. The RT-qPCR results revealed that all 11 of the predicted ORFs could be transcribed to corresponding mRNAs. As shown in Fig. 2, among the 11 ORFs, *orf9* and *orf10* are characterized by the highest transcription levels. These two genes are predicted to be responsible for the metabolism of propionicin SM1. Additionally, the high expression levels of *orf9* and *orf10* may

TABLE 3 Putative ORFs of plasmid pZGX01 from *P. acidipropionici* ATCC 4875

ORF	Start position	Stop position	Residue of the protein	Potential function of the putative protein	Highest homology	% amino acid similarity
ORF1	623	18	302	Double-stranded DNA binding protein	pKNR01 (*Rhodococcus opacus*)	52
ORF2	1075	1308	77	DNA binding domain protein	RHH_1 (*Kytococcus sedentarius*)	68
ORF3	1292	1555	87	DNA binding protein	RelE (*Mobiluncus mulieris*)	54
ORF4	2258	1620	212	Recombinase	ORF3 (*Rhodococcus erythropolis*)	34
ORF5	2483	2316	55	None	None	
ORF6	2696	3604	302	Replicase	*repA* of pMEC2 (*Micrococcus luteus*)	64
ORF7	3604	3948	114	Replication initiation protein	*repB* of pRGO1 (*Propionibacterium acidipropionici*)	100
ORF8	3945	4472	175	None	None	
ORF9	4971	5594	207	Propionicin SM1	PpnA of pLME106 (*Propionibacterium jensenii*)	100
ORF10	5647	5973	108	None	None	
ORF11	6132	6677	181	Resolvase	PinR (*Propionibacterium acidipropionici*)	99

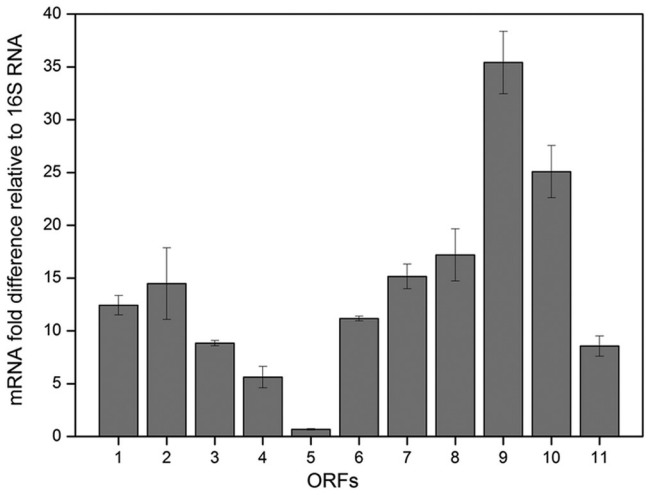

FIG 2 The transcription level relative to 16S RNA of 11 predicted ORFs from pZGX01. The total RNA extracted from cells was reverse transcribed to cDNA. The obtained cDNA was subjected to quantitative PCR, and 16S RNA was set as the guide sample.

benefit the survival of the strain. The transcription levels of *orf1*, *orf2*, *orf3*, *orf6*, *orf7*, and *orf8* are at intermediate levels, and their encoding proteins seem to be involved in DNA binding and replication, which are important for plasmid maintenance. *orf4*, *orf5*, and *orf11* have relatively low transcription levels.

Shuttle vector construction and transformation. A shuttle vector between *Propionibacterium* and *E. coli* was constructed. A large fragment containing *orf2* to *orf10* was obtained from pZGX01 by digestion with EcoRI and BamHI and ligated to EcoRI-BamHI-digested *E. coli* pUC18. The resulting pZGX01-1 plasmid was digested with NheI and self-ligated, yielding the pZGX01-2 plasmid. A 1.5-kb PCR product carrying the *cmx*(A) (chloramphenicol resistance) gene and *cml*(A) genes was ligated to pZGX01-3, which was obtained by digesting pZGX01-2 with StuI (inside *orf2*), yielding the shuttle vector pZGX04 (Fig. 3). The GC content of *cmx*(*A*) is 63%, which is consistent with the GC content of *Propionibacterium*. Since it was demonstrated that the expression level of the antibiotic resistance gene with high GC content was good in *Propionibacterium* (21), this consistency is particularly important.

The pZGX04 plasmid cloned in *E. coli* JM110 was used to

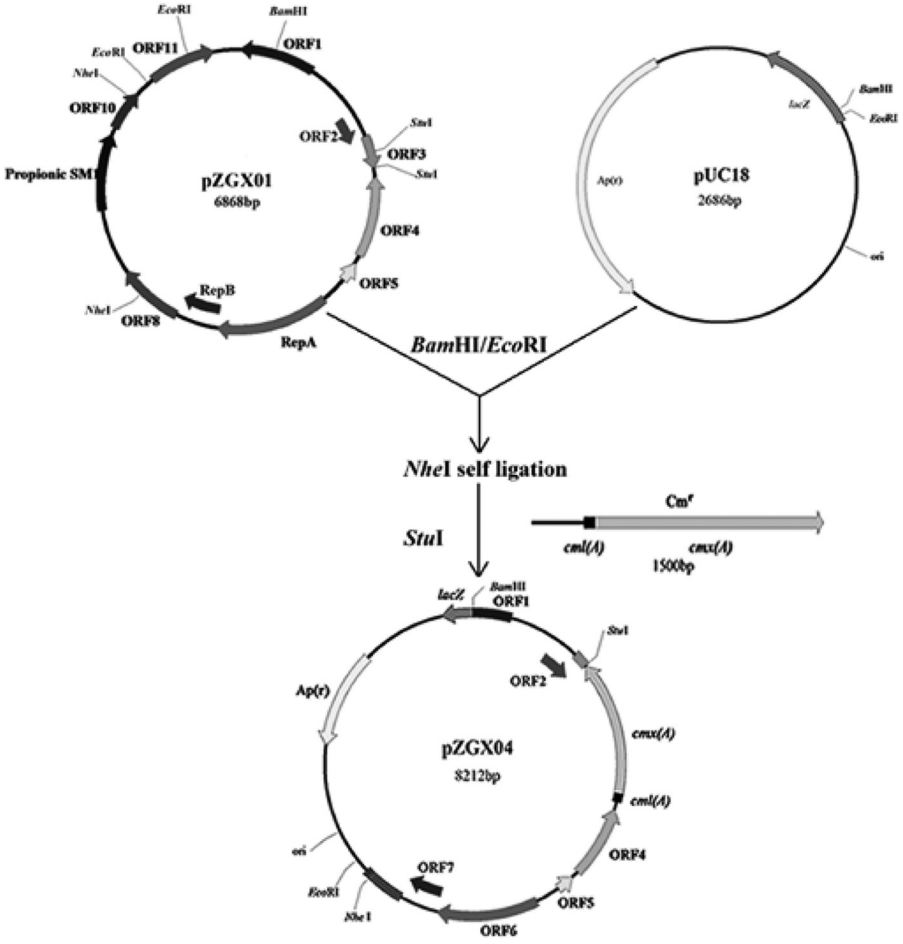

FIG 3 Scheme for vector pZGX04 construction. A large fragment containing *orf2* to *orf10* was obtained from pZGX01 by digestion with EcoRI and BamHI, and the fragment was ligated to EcoRI-BamHI-digested *E. coli* plasmid pUC18. The resulting plasmid was digested with NheI and self-ligated. Then, the resulting plasmid was digested with StuI (inside *orf2*) and ligated to a 1.5-kb PCR product carrying the *cmx*(A) and *cml*(A) genes, yielding the shuttle vector pZGX04. Cm^r, gene encoding chloramphenicol-resistant protein; Ap^r, gene encoding ampicillin-resistant protein; RepA, gene encoding Rep A protein; RepB, gene encoding Rep B protein; *lacZ*, β-galactosidase; ori, colE replication origin.

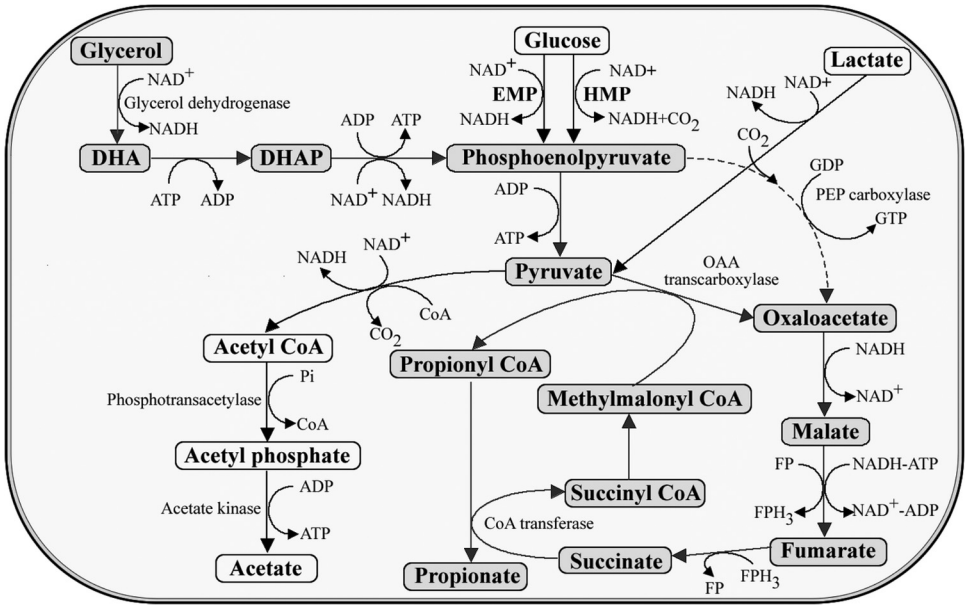

FIG 4 Synthesis pathways for propionic acid from glycerol, glucose, and lactate in *Propionibacterium*. The biosynthetic pathway from glycerol to propionic acid proceeds from glycerol to dihydroxyacetone (DHA) to dihydroxyacetone phosphate (DHAP) to phosphoenolpyruvate to pyruvate and oxaloacetate and from oxaloacetate to malate, fumarate, succinate, succinyl coenzyme A (CoA), methylmalonyl CoA, propionyl CoA, and propionate (see arrows). Acetate is the main byproduct in propionic acid production. Gray color indicates the biosynthetic pathway from glycerol to propionic acid.

transform *P. jensenii* ATCC 4868. The growth phase for preparation of competent cells, electroporation resistance, voltage, and amount of plasmid DNA have been optimized. It was found that when the competent cells were prepared at a late exponential phase (OD_{600} = 0.7) and 1.5 μg of pZGX04 was exposed to an electric field strength of 12.5 kV/cm with a resistance of 200 Ω, the transformation efficiency reached a maximum of 30 to 40 CFU/μg DNA.

Transformation host range and stability. To further examine the transformable capacity of this shuttle vector in other *Propionibacterium* species, *P. acidipropionici* ATCC 4875, *P. acidipropionici* ATCC 4965, *P. acidipropionici* ATCC 25562, *P. jensenii* ATCC 4868, *P. jensenii* ATCC 4870, *P. freudenreichii* subsp. *freudenreichii* ATCC 6207, *P. freudenreichii* subsp. *shermanii* ATCC 9614, *P. thoenii* ATCC 4872, and *P. thoenii* ATCC 4874 (Table 1) have been tested. Interestingly, it was found that strains *P. jensenii* ATCC 4870 and *P. freudenreichii* subsp. *freudenreichii* ATCC 6207 could also be transformed by pZGX04 with transformation efficiencies of 25 to 30 CFU/μg DNA and 30 to 40 CFU/μg DNA, respectively.

The *Propionibacterium* transformants containing pZGX04 were cultured for 30 generations in medium without chloramphenicol and were then spread on SLB agar plates with and without chloramphenicol. Only a few differences in the number of colonies were observed, indicating that the vector was segregationally stable in all the tested organisms (≤9% loss after 30 generations without selection). Moreover, sequencing of the reisolated vector from *P. jensenii* also verified that the vector could be replicated in *Propionibacterium* without reconstruction (data not shown).

Expression of the *gldA* gene in *P. jensenii* ATCC 4868. Glycerol dehydrogenase is an enzyme involved in the PA biosynthesis pathway of *Propionibacterium* and plays an important role during the assimilation of glycerol as the carbon source (Fig. 4). Here, we attempted to express the glycerol dehydrogenase-encoding gene (*gldA*) from *K. pneumoniae* within the constructed vector to improve the production of PA by *P. jensenii* ATCC 4868. The original promoter of pZGX04 was kept in the construction of the expression vector and was used to express *gldA*. A PCR product of pZGX04 using primers pZGX04-FWD and pZGX04-REV was ligated to another PCR product of the *K. pneumoniae* subsp. *pneumoniae* ATCC 12657 genome using primers GLD-FDW and GLD-REV. The resulting vector (named pZGX04-*gldA*) was transformed into *P. jensenii* ATCC 4868. The RT-qPCR analysis revealed that the *gldA* transcription level was 8.07-fold of that of the homologous gene (*glpA*) in the wild-type *P. jensenii* ATCC 4868.

Figure 5 shows the effects of the *gldA* expression on PA production by *P. jensenii* ATCC 4868. In batch fermentation, the engineered strain *P. jensenii* ATCC 4868 (pZGX04-*gldA*) displays a much higher glycerol dehydrogenase (GDH) activity than the wild-type *P. jensenii* ATCC 4868 (Fig. 1), and it consumes glycerol more rapidly. Moreover, PA production (amount of PA produced) with the expression of *gldA* was increased from 22.06 ± 1.2 g/liter to 28.23 ± 1.0 g/liter and the productivity (production per hour) from 0.15 $g \cdot liter^{-1} \cdot h^{-1}$ to 0.20 $g \cdot liter^{-1} \cdot h^{-1}$. However, the DCW of engineered *P. jensenii* ATCC 4868 (pZGX04-*gldA*) decreased, indicating that the plasmid transformation slows cell growth. *P. jensenii* ATCC 4868 (pZGX04-*gldA*) also has a higher $Y_{P/S}$ (mass ratio of PA to succinic acid), $Y_{P/A}$ (mass ratio of PA to acetic acid), and $Y_{PA/Glycerol}$ than the wild-type strain.

The results indicate that the expression of *gldA* enhances the PA yield and reduces the concentration of byproducts (acetic acid and succinic acid) under anaerobic conditions. The expression of the *gldA* gene increases the transformation rate of glycerol to dihydroxyacetone (DHA) and thus generates more NADH (Fig. 4).

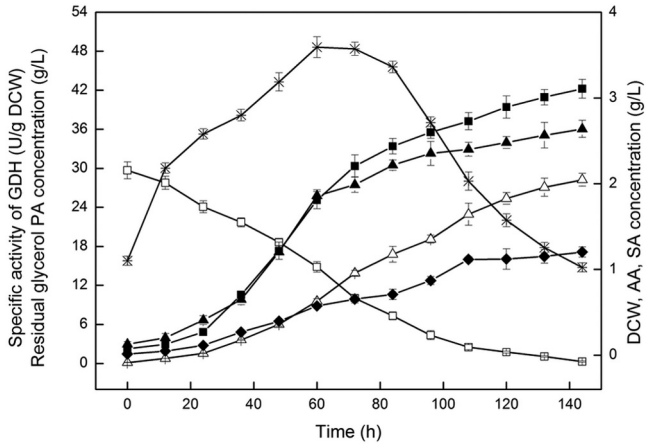

FIG 5 Batch fermentation kinetics of propionic acid production from glycerol with *P. jensenii* ATCC 4868 (pZGX04-*gldA*) at pH 7.0 and 30°C for 144 h. Batch fermentations were performed under anaerobic conditions (nitrogen gassing) in a 3-liter Bioreactor containing 2 liters of culture medium supplemented with 30 g/liter glycerol. Samples were taken from the fermentor every 12 h. △, propionic acid (PA); □, residual glycerol; ▲, dry cell weight (DCW); ■, acetic acid (AA); ◆, succinic acid (SA); *, specific activity of GDH.

Since the synthesis pathway of PA is a NADH consumption process, the accumulation of NADH is considered a favorable event. Although the expression of *gldA* induced slower cell growth during the batch fermentation, the specific PA synthesis rate results clearly improved. Overall, these findings indicate that *gldA* expression can noticeably increase the PA titer, productivity, and yield.

Conclusions. In summary, our results show that the *P. jensenii* ATCC 4868 strain is a good candidate for PA production. Thus, we developed a shuttle vector for the metabolic engineering of *P. jensenii* ATCC 4868 to further improve PA production. The constructed vector was successfully transformed into *P. jensenii* ATCC 4868. The report showing that a vector can be transformed into *P. jensenii* with a high stability. The constructed shuttle vector was also used to efficiently engineer *P. freudenreichii*. The glycerol dehydrogenase-encoding gene (*gldA*) was expressed in *P. jensenii* ATCC 4868 by the shuttle vector, resulting in a 26.07% increase in the PA production. We believe that the developed shuttle vector could strengthen the engineering capacity of *P. jensenii* and further improve its production potential by vector-based metabolic engineering strategies.

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