Appl Microbiol Biotechnol (2014) 98:8963–8973
DOI 10.1007/s00253-014-5824-2

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

A markerless gene replacement method for *B. amyloliquefaciens* LL3 and its use in genome reduction and improvement of poly-γ-glutamic acid production

Wei Zhang · Weixia Gao · Jun Feng · Chi Zhang · Yulian He · Mingfeng Cao · Qiang Li · Yang Sun · Chao Yang · Cunjiang Song · Shufang Wang

Received: 19 March 2014 / Revised: 20 April 2014 / Accepted: 10 May 2014 / Published online: 25 May 2014
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Abstract We herein adapted a markerless gene replacement method by combining a temperature-sensitive plasmid pKSV7 with a counterselectable marker, the *upp* gene encoding uracil phosphoribosyltransferase (UPRTase), for the poly-γ-glutamic acid (γ-PGA)-producing strain *Bacillus amyloliquefaciens* LL3. Deletion of the *upp* gene conferred LL3 5-fluorouracil (5-FU) resistance. Sensitivity to 5-FU was restored when LL3 Δ*upp* was transformed with pKSV7-based deletion plasmid which carries a functional allele of the *upp* gene of *Bacillus subtilis* 168. These observations allowed us to adapt a two-step plasmid integration and excision strategy to perform markerless deletion of genes of interest. Deletion plasmid harboring a mutant allele of the target gene was first integrated in the genome by culturing cells under nonpermissive conditions for pKSV7 replication. Single-crossover recombinants were then grown without antibiotics to aid the second recombinational event. 5-FU was used to select for double-crossover recombinants with plasmid evicted from the chromosome. The resulting recombinants either harbored the wild-type or mutated allele of the target gene and could be identified by PCR and DNA sequencing. Using this method, we successively removed the *amyA* gene and a 47-kb fragment of the *bae* cluster from the genome of LL3, with higher efficiency compared with previous reports. We also investigated the effects of a transcriptional regulator, RocR, on γ-PGA production and cell growth. Specific γ-PGA production of the *rocR* mutant was increased by 1.9-fold, which represents a new way to improve γ-PGA production.

Keywords *Bacillus amyloliquefaciens* · Markerless gene replacement · Genome reduction · Gamma-PGA · RocR

W. Zhang · W. Gao · J. Feng · C. Zhang · Y. He · Q. Li · Y. Sun · C. Yang · C. Song
Key Laboratory of Molecular Microbiology and Technology for Ministry of Education, College of Life Sciences, Nankai University, Tianjin, People's Republic of China

W. Zhang · J. Feng · C. Song (✉) · S. Wang (✉)
State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences, Nankai University, Tianjin, People's Republic of China
e-mail: songcj@nankai.edu.cn
e-mail: wangshufang@nankai.edu.cn

M. Cao
Department of Chemical and Biological Engineering, Iowa State University, Ames, IA, USA

Introduction

Bacillus amyloliquefaciens strains are important microbial cell factories for the production of many industrial enzymes, such as α-amylase, levansucrase, and fibrinolytic enzymes, and other products, like purine nucleosides and riboflavin for example (Gangadharan et al. 2008; Rairakhwada et al. 2010; Sauer et al. 1998; Xu et al. 2013). *B. amyloliquefaciens* LL3 is a glutamic acid-independent poly-γ-glutamic acid (γ-PGA)-producing strain, isolated from traditional fermented food, natto (Cao et al. 2010, 2011). γ-PGA is a promising, environmentally friendly biomaterial composed of L- and D-glutamic acids linked by γ-amide linkages, with outstanding water solubility, biocompatibility, and degradability (Shih and Van 2001) and with a wide range of applications ranging from hydrogels, flocculants, drug delivery, cosmetics to feed additives (Sung et al. 2005).

The common routes to improving microbial γ-PGA production include the identification of new producer strains with higher γ-PGA yields and the optimization of fermentation conditions (Bajaj et al. 2009; Shi et al. 2006). Few studies have attempted to improve microbial γ-PGA production by genetic engineering. Deletion of *epsA*, a gene responsible for

the synthesis of exopolysaccharides (EPS), was reported to cause γ-PGA overproduction in *B. amyloliquefaciens* C06, probably because of redistributing metabolic fluxes (Liu et al. 2011). By knocking out the genes encoding the major γ-PGA-degrading enzymes, *ggt* and *pgdS* in *Bacillus subtilis* 168, γ-PGA yield was improved to a level similar to the top wild-type γ-PGA producer strains (Scoffone et al. 2013). However, it is imperative to identify more genes to further improve γ-PGA production. One possible method is through large-scale genome reduction. During the construction of *B. subtilis* MGB874, a sequential deletion of 865 genes was performed from *B. subtilis* 168. The resulting strain MGB874 shows enhanced enzyme production, partly because of the deletion of *rocR*, which is supposed to increase the flux from 2-oxoglutarate to glutamate, and then lead to increased syntheses of the other amino acids via transamination (Morimoto et al. 2008; Manabe et al. 2011). Considering that the intracellular glutamate is the only source for γ-PGA synthesis during the fermentation of *B. amyloliquefaciens* LL3 (Wu et al. 2008; Geng et al. 2011), deletion of *rocR* may also increase γ-PGA production.

To increase γ-PGA yield and to gain a better understanding of the mechanisms in γ-PGA synthesis, an efficient gene replacement method is required for *B. amyloliquefaciens* LL3 and other strains. In particular, a markerless gene deletion or insertion method is essential for metabolic engineering and synthetic biology applications, because mutations have to be accumulated in one single strain, and polar effects on downstream regions should be avoided (Datsenko and Wanner 2000; Zhang et al. 1998). However, some *B. amyloliquefaciens* strains cannot develop natural competence, compared with their close relative *B. subtilis*, which allows incorporation of DNA and subsequent integration into the chromosomes (Fabret et al. 2002). For *B. amyloliquefaciens* strains with low transformation efficiency, temperature-sensitive plasmids or a very recently reported transconjugative plasmid system can be used to perform gene deletion or insertion, because one single transformant is enough for subsequent selection (Okibe et al. 2011; Zakataeva et al. 2010; Rachinger et al. 2013). When we performed gene replacement experiments in *B. amyloliquefaciens* LL3 using pKSV7 without a counterselectable marker, however, the method is inefficient because of the interference of stains that had not undergone the second homologous recombinational events. Even among the double-crossover mutants, strains restored to the wild-type would dominate when the target genes are not readily removed from the genome. Thus, the lack of a counterselectable marker often leads to extensive screening. The counterselectable marker becomes more important in genome-scale applications. In *B. subtilis*, the gene encoding uracil phosphoribosyl-transferase (UPRTase), *upp*, was reported as a counterselectable marker to select for the removal of the inserted selection marker. Pyrimidine analog 5-fluorouracil (5-FU) can be converted into 5-fluoro-UMP by UPRTase and then metabolized into 5-fluoro-dUMP, a toxic substrate for the cell. PCR fragments comprising homologous arms, an antibiotic marker, and an ectopic copy of *upp* gene were assembled by overlapping PCR and then transformed into a UPRTase-deficient background host. Double-crossover mutants in which the inserted antibiotic marker has been evicted from the genome can be recovered from agar plates supplemented with 5-FU (Fabret et al. 2002). Since then, *upp* has been proven to be a powerful counterselectable marker in other bacteria, such as *Enterococcus faecalis* (Kristich et al. 2005), *Lactobacillus acidophilus* (Goh et al. 2009), and *Desulfovibrio vulgaris* (Keller et al. 2009). Moreover, at least 16 *B. amyloliquefaciens* strains contain a copy of *upp* gene (data not shown). Therefore, we choose *upp* to construct our gene replacement system.

We previously reported the chromosomal insertion of the *Vitreoscilla* hemoglobin gene *vgb*, using a temperature-sensitive plasmid pKSV7, which left an antibiotic-resistant marker in the genome and was inefficient (Zhang et al. 2013). In this paper, we combined the temperature-sensitive plasmid pKSV7 with the frequently used counterselectable marker, *upp*, to perform markerless gene modification in naturally nontransformable strain *B. amyloliquefaciens* LL3. Using this method, we successfully removed a 47-kb DNA fragment from the genome at one time. We also demonstrated the efficiency of the *upp* counterselection scheme by performing in-frame deletion of the gene encoding a transcriptional regulator, *rocR*, to uncover its effects on γ-PGA production. This method may also be applicable to other *B. amyloliquefaciens* strains.

Materials and methods

Strains and growth conditions

The strains used in this study are listed in Table 1. *Escherichia coli* DH5α was used for plasmid construction. The *dam*, *dcm*-deficient *E. coli* GM2163 was used to prepare the unmethylated plasmids. *E. coli* strains were grown in Luria–Bertani (LB) medium at 37 °C with aeration. Recombinant plasmids were propagated in *E. coli* strains in the presence of 100 μg/mL ampicillin. *B. subtilis* 168 was the source of the *upp* cassette. *Bacillus* strains were grown at 37 °C, except during plasmid transformation (30 °C) and integration/excision (42 °C) experiments. The *B. amyloliquefaciens* LL3 strain was deposited in the China Center for Type Culture Collection (CCTCC) with accession number CCTCC M 208109 (Geng et al. 2011). The fermentation medium consisted of sucrose 50 g/L, $(NH_4)_2SO_4$ 2 g/L, $MgSO_4$ 0.6 g/L, KH_2PO_4 6 g/L, K_2HPO_4 14 g/L, and 2 mL mineral elements including 1 mM $FeSO_4·4H_2O$, $CaCl_2·2H_2O$, $MnSO_4·4H_2O$, and $ZnCl_2$ (pH 7.2). When required, media were supplemented with ampicillin (Ap; 100 μg/mL), chloramphenicol (Cm; 5 μg/mL), or tetracycline

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description	Source or literature
B. amyloliquefaciens strains		
LL3	Glutamic acid independent poly-γ-glutamic acid (γ-PGA)-producing strain	Geng et al. (2011)
LL3 Δ*upp*	LL3 carrying an in-frame deletion in the *upp* gene	This study
LL3 Δ*upp* Δ*amyA*	LL3 Δ*upp* carrying an in-frame deletion in the *amyA* gene	This study
LL3 Δ*upp* Δ*amyA* Δ*bae*	LL3 Δ*upp* Δ*amyA* carrying a ~47-kb deletion in part of the bacillaene synthesis operon	This study
LL3 Δ*upp* Δ*rocR*	LL3 Δ*upp* carrying an in-frame deletion in the *rocR* gene	This study
B. subtilis 168	*trpC2*, source of the *upp* cassette	ATCC
E. coli strains		
DH5a	*supE44* Δ*lacU169*(_*80 lac*ZΔM15) *recA1 endA1 hsdR17*(rK−mK$^+$) *thi-1gyrA relA1* F−Δ(*lacZYA-argF*)	Tiangen
GM2163	F$^-$*dam-13::*Tn*9* (Cam^r) *dcm-6 hsdR2* ($r_k^-m_k^+$) *leuB6 hisG4 thi-1 araC14 lacY1 galK2 galT22 xylA5 mtl-1 rpsL136* (Str^r) *fhuA31 tsx-78 glnV44 mcrA mcrB1*	Fermentas
Plasmids		
pKSV7	*E. coli–Bacillus* shuttle vector, colE1, pE194ts, Ap^r, Cm^r	Smith and Youngman (1992)
pWH1520	*E. coli–Bacillus* shuttle vector, Ap^r, Tet^r	Zhang et al. (2013)
pKSV7-upp	pKSV7 derivative carrying a mutant copy of the *upp* gene used for deletion of the *upp* gene	This study
pKSU	pKSV7 derivative, carrying an ectopic copy of *upp* gene from *B. subtilis* 168	This study
pKSU-amyA	pKSU derivative, carrying homologous arms for the deletion of the *amyA* gene	This study
pKSU-bae	pKSU derivative, used for the deletion of the partial *bae* operon	This study
pKSU-rocR	pKSU carrying a mutant copy of *rocR* gene	This study
pWH-rocR	Complementation plasmid for the *rocR* mutant, pWH1520 derivative	This study

(Tc; 10 μg/mL). Stock solutions (75 mg/mL) of 5-FU were prepared in DMSO and added into the media at a final concentration of 1.3 mM.

DNA isolation and manipulation

The primers used in this study are listed in Table 2. Overlapping PCR was performed to obtain homologous arms. DNA polymerases, digestion enzymes, and T4 DNA ligase were purchased from Takara (Dalian, China). PCR, enzyme digestion, and ligation reactions were performed as recommended by the enzyme suppliers. DNA fragments were analyzed on 0.8 % agarose gels and purified using an Axygen gel DNA recovery kit (Axygen, CA, USA). Genomic DNA was isolated using a TIANamp Bacteria DNA kit (TIANGEN, Beijing, China). For the transformation of LL3 and its derivative mutants, plasmids were first transformed into the adenine and cytosine methylation-deficient strain, *E. coli* GM2163, and then treated with BamHI methyltransferase (New England Biolabs, MA, USA) according to the manufacturer's instructions, to prevent potential digestion by the restriction-modification systems of LL3.

Bacterial transformation

Competent *E. coli* cells were purchased from Transgen Biotech (Beijing, China), and transformation was performed according to the manufacturer's instructions. All the plasmids were transformed into *B. amyloliquefaciens* LL3 using the high osmolarity electroporation method (Xue et al. 1999; Zhang et al. 2011), with modifications as described previously (Zhang et al. 2013).

Construction of LL3 Δ*upp*

All the deletions (except *bae*) carried out in this study were based on the rule of in-frame deletion. The homologous arms were obtained by joining up- and downstream fragments flanking the deletion region through overlapping PCR, such that no additional sequence was left in the genome after deletion. The DNA fragments flanking the *upp* gene were PCR-amplified using the LL3 chromosome as a template. The 500-bp upstream fragment was obtained using primers UppUP-F and UppUP-R and the 500-bp downstream fragment using primers UppDN-F and UppDN-R. The 5′-end of UppUP-R contained a sequence complementary to UppDN-F (25 bases at least), allowing the joining of these two fragments in a subsequent PCR reaction. The resulting fragment was digested with *Eco*RI/*Kpn*I and ligated to a similarly digested pKSV7 to generate pKSV7-upp. The remaining open reading frame (ORF) in the deletion plasmid contained 31 N-terminal and 17 C-terminal amino acids of the intact protein. pKSV7-upp was transformed into LL3 by electroporation, and Cm^R transformants were selected and cultivated overnight in LB

Table 2 Primers used in this study

Primer names	Sequence (5′ to 3′)[a]
UppUP-F	CAGC**GAATTC**ACGTCATCTCGAACGCGAAG
UppUP-R	GACCCGGAACGATCATACTGTGTTCAGCTCC
UppDN-F	TGAACACAGTATGATCGTTCCGGGTCTCGGTG
UppDN-R	GCGC**GGTACC**CACAAATACATATCTCCGTTG
UppOUT-F	GAGGACATCGCCCGCGACC
UppOUT-R	GACAAAATCAACAATCCATTCCATG
Upp-F	ACA**GGTACC**GATCCTAAAACCCGCTTG
Upp-R	TGA**GGATCC**TTATTTTGTTCCAAACATGCGGTC
AmyUP-F	GCGC**TCTAGA**GCCTGAAATTAAAAAGCTGGC
AmyUP-R	ACATAAATGGAGACCACAAGTCTGAACGAAAC
AmyDN-F	CGTTCAGACTTGTGGTCTCCATTTATGTTCAG
AmyDN-R	CGAG**TCTAGA**TTGTTGAAGGCAAATATCTG
AmyOUT-F	TCTCAGCGGAAAAAGAATCATC
AmyOUT-R	GCTTATTTCGACCAGCTGATTC
BaeUP-F	CGG**TCTAGA**AAACTACATGTCATCTGTCATTAACG
BaeUP-R	CATCGAGAAGTTCTTAAAAGATCCGGGCAGAC
BaeDN-F	CCCGGATCTTTTAAGAACTTCTCGATGCCTAC
BaeDN-R	TGA**GTCGAC**GTGACGGCTTCTCTTTCAG
BaeOUT-F	ATGATACCGCTCCATGTCAGCTCACTTG
Bae-M	GGCAGCGCAATGTAGCCCTTGAC
BaeOUT-R	CGCCGTGCTTCGTTCATCTAATTCG
RocRUP-F	AGTT**GGATCC**ACTTCCGTTTAATCCCCAG
RocRUP-R	ATCCGCTCCAGCCCGGAAACGCAAAAAAATTTTG
RocRDN-F	TTTTTTGCGTTTCCGGGCTGGAGCGGATGAAATG
RocRDN-R	TATA**GTCGAC**GCCGGCGGAAAAATTCAC
RocROUT-F	AGGGAGGTCATATCCCAGGCCGGTC
RocROUT-R	TAAGTCAGGAAGGGCTGTCCGTGCG
RocR-F	AGCA**GGTACC**GATTCCCCGCCTTTCTTC
RocR-R	TCGA**GGATCC**ACCAGAATGACAAATTCAGG

[a] Restriction sites used for the cloning of PCR amplicons are indicated in bold

with chloramphenicol at 30 °C and then transferred at 42 °C to facilitate plasmid integration. The culture was then spread on LB agar plates with chloramphenicol and incubated overnight at 42 °C. Primers UppOUT-F and UppOUT-R (corresponding to p1 and p2 in Fig. 1, respectively) were designed according to the genome sequence flanking the homologous arms and were used in both single- and double-crossover recombinants screen. In single-crossover strains, the plasmid (approximately 8,000 bp) was integrated into the genome of LL3 through homologous recombination involving either of the homologous arms, such that the PCR product of UppOUT-F and UppOUT-R would be approximately 9,000 bp. The PCR product using wild-type LL3 as a template, however, would be about 1,000 bp. The verified single-crossover recombinant was cultured in LB without chloramphenicol for more than 30 generations (usually with two transfers) to facilitate the second recombinational event. The culture was then plated on LB agar plates containing 1.3 mM 5-FU and incubated at 42 °C for 18 h. The chromosomal structure at the *upp* locus of several $5FU^R$ colonies was analyzed using primers UppOUT-F/UppOUT-R and DNA sequencing. One colony carrying the correct deletion was chosen and used as the host strain for all the subsequent deletions.

Construction of the *upp* cassette

An 850-bp DNA fragment carrying the *upp* gene with its 5′ regulatory region and its 3′ transcription terminator was generated by PCR from *B. subtilis* 168 genomic DNA using primers Upp-F and Upp-R. After digesting with *Kpn*I and *Bam*HI, the fragment was cloned in the *Kpn*I–*Bam*HI sites of pKSV7. The resulting counterselective plasmid was designated pKSU.

Construction of deletion plasmids

The ~500-bp up- and downstream homologous arms for *amyA* deletion were obtained using primer pairs AmyUP-F/AmyUP-R and AmyDN-F/AmyDN-R, respectively, and spliced by a subsequent overlapping PCR. After digestion by *Xba*I, the fragment was ligated to a similarly digested pKSU to generate pKSU-amyA. The remaining ORF in the deletion plasmid contained 14 N-terminal and eight C-terminal amino acids of the intact AmyA protein. The deletion plasmids pKSU-bae and pKSU-rocR for *bae* and *rocR* deletion, respectively, were similarly constructed. The remaining ORF in pKSU-rocR contained 23 N-terminal and seven C-terminal amino acids of the intact RocR protein. For *bae* deletion, regions from *baeB* to *baeL* as well as 11 bp of *bamM* were targeted (corresponding to 1,745,468–1,792,823 in LL3 genome). The up- and downstream homologous arms for *bae* and *rocR* deletion were 1.5 and 0.5 kb, respectively. The resulting deletion plasmids were used to transform LL3 Δ*upp*-competent cells. The selection procedure was described in detail in the "Results" section.

Starch degradation assay

Overnight cultures of LL3 Δ*upp* and LL3 Δ*upp* Δ*amyA* in LB liquid were added in the Oxford cups placed in LB agar containing 2 % soluble starch and cultured at 37 °C for another 24 h. The plates were then covered by iodine solution to examine the degradation of starch.

Complementation experiments of LL3 Δ*upp* Δ*rocR*

A 1,970-bp DNA fragment carrying the *rocR* gene with its 5′ regulatory region and its 3′ transcription terminator was

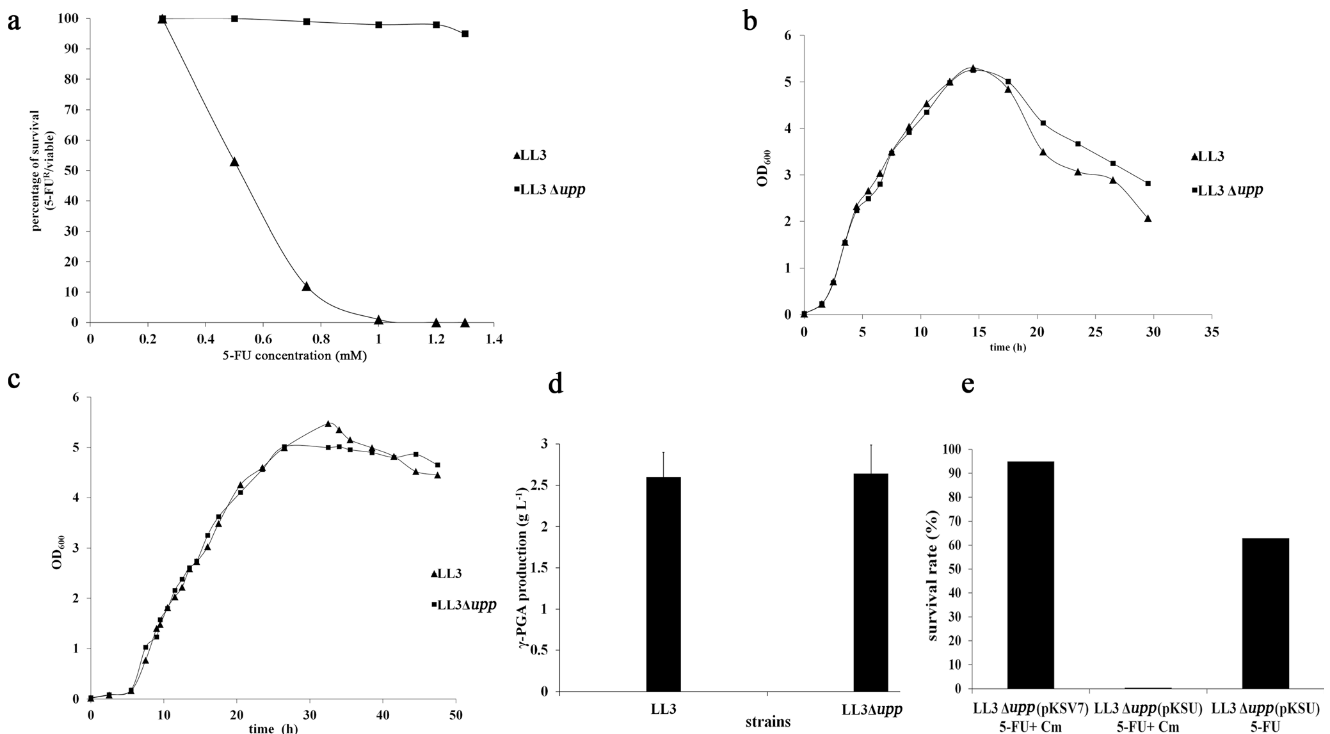

Fig. 1 Growth and γ-PGA production profiles of LL3 and LL3 Δ*upp*. **a** Cultures of wild-type LL3 (*triangle*) and LL3 Δ*upp* (*square*) were plated on LB agar plates supplemented with different concentrations of 5-FU and grown at 37 °C for 16 h before counting colonies. The proportion of 5-FU^R colonies was plotted as a function of 5-FU concentration. The result presented was one of the triplicate replicates, which was similar with the other two **b** growth characteristics of LL3 (*triangle*) and LL3 Δ*upp* (*square*) in LB. **c** Growth characteristics of LL3 (*triangle*) and LL3 Δ*upp* (*square*) in the fermentation medium. The results presented for growth experiments are the means of three independent replicates, and the standard deviations are below 10 %. **d** γ-PGA production from LL3 and LL3 Δ*upp*. *Error bars* represent standard deviations ($n = 3$). **e** Complementation of the *upp* mutant. LL3 Δ*upp* was transformed with pKSV7 and pKSU to obtain strain LL3 Δ*upp* (pKSV7) and LL3 Δ*upp* (pKSU), respectively. Overnight cultures of the two strains were plated on LB agar containing 5 μg/mL Cm or 1.3 mM 5-FU or both Cm and 5-FU and grown at 30 °C for 24 h before counting colonies. Survival rates were calculated against the colony numbers from plates containing Cm only. The result presented was one of the triplicate replicates, which was similar with the other two. Introduction of pKSU, which carries the *upp* gene from *B. subtilis* 168, restored the sensitivity of LL3 Δ*upp* to 5-FU. LL3 Δ*upp* (pKSU) showed a high survival rate on LB agar containing 5-FU only because of the loss of the plasmid

generated by PCR from LL3 chromosomal DNA using primers RocR-F and RocR-R. After digesting with *Kpn*I and *Bam*HI, the fragment was cloned in the *Kpn*I–*Bam*HI sites of pWH1520. The resulting plasmid was designated pWH-rocR and was transformed in LL3 Δ*upp* Δ*rocR*.

Cell growth and γ-PGA production in shake flasks

For growth experiments, *B. amyloliquefaciens* LL3 and its derivatives were first grown overnight in test tubes containing LB liquid medium and then inoculated into fresh LB or fermentation medium to an optical density at 600 nm (OD_{600}) of approximately 0.05–0.1, and growth was monitored at OD_{600}. For fermentation experiments, *B. amyloliquefaciens* strains were first grown for 16 h in LB at 37 °C in a shaking incubator and then diluted 20-fold into 500-mL shake flasks containing 100 mL fermentation medium and incubated at 37 °C with shaking for 48 h. Then the fermentation broth was centrifuged at 8,000 × g (4 °C) for 20 min. The cell pellet was washed three times with dH_2O and then dried and weighed. γ-PGA was extracted using an ethanol precipitation method as previously described (Zhang et al. 2013). Experiments were independently repeated at least three times and the mean and standard deviations were calculated.

Results

Construction and characterization of *B. amyloliquefaciens* LL3 Δ*upp*

Overnight cultures of wild-type LL3 was serially diluted and plated on LB agar supplemented with different concentrations of 5-FU or an equivalent amount of DMSO, to determine its sensitivity to 5-FU. Cell growth was completely inhibited in the medium containing 1.3 mM 5-FU, while the presence of DMSO showed no effects

(Fig. 1a). A final concentration of 1.3 mM 5-FU was thus applied in all subsequent double-crossover mutants screening. For *upp* deletion, colonies recovered from plates containing 5-FU were verified by PCR and DNA sequencing and tested for Cm sensitivity. All colonies grew well in the medium supplemented with 1.3 mM 5-FU and were sensitive to Cm (data not shown), indicating excision of the plasmid from the chromosome. One correct colony was designated LL3 Δ*upp* and compared with wild-type LL3. Growth and fermentation experiments indicated that LL3 Δ*upp* showed no apparent differences in cell growth and γ-PGA production (Fig. 1b–d).

Construction of the counterselectable deletion plasmid

As the promoter region of the *upp* gene of *B. amyloliquefaciens* LL3 was not identified, the *upp* cassette of *B. subtilis* 168 was PCR-amplified and ligated to pKSV7. The resulting counterselectable plasmid was designated pKSU. LL3 Δ*upp* was transformed with pKSV7 and pKSU, respectively. LL3 Δ*upp* (pKSV7) grew well in LB supplemented with 5-FU and Cm, whereas LL3 Δ*upp* (pKSU) showed no growth (Fig. 1e). Thus, the *upp* gene from *B. subtilis* 168 could function well in *B. amyloliquefaciens* LL3, at least in the form of a plasmid. This laid the foundation of our counterselection scheme.

Rationale for the markerless gene replacement method

All deletion constructions were performed as shown in Fig. 2. The deletion plasmid was first constructed by ligating a mutant copy of a certain gene or region to pKSU and then transformed in LL3 Δ*upp* or its derivatives at 30 °C. Transformants were then cultured at 42 °C with Cm to facilitate plasmid integration. Primers p1 and p2 were designed to anneal to the sequence flanking the homologous arms in the genome, and p3 was designed according to the sequence of the deletion fragment. The single-crossover recombinants were identified as that in the construction of the Δ*upp* mutant using primer pairs p1/p2 or p1/p3 and cultured at 42 °C without Cm to facilitate plasmid excision. The culture was then plated on LB agar plate containing 5-FU. Double-crossover mutants obtained from LB agar with 5-FU comprised two circumstances: restoration to wild type or gaining the desired deletion. Both kinds of strains were resistant to 5-FU and were sensitive to chloramphenicol because of the excision of the plasmid from the chromosome. PCR with primers p1 and p2 could distinguish them. The strains with the desired mutation would produce a shorter product than the wild type. For long fragments, up to 50 kb for example, the strain restored to wild type would not generate any product. PCR performed with p1 and p3 could exclude wild-type strains that might be mixed with the desired mutation. All deletions were verified by DNA sequencing.

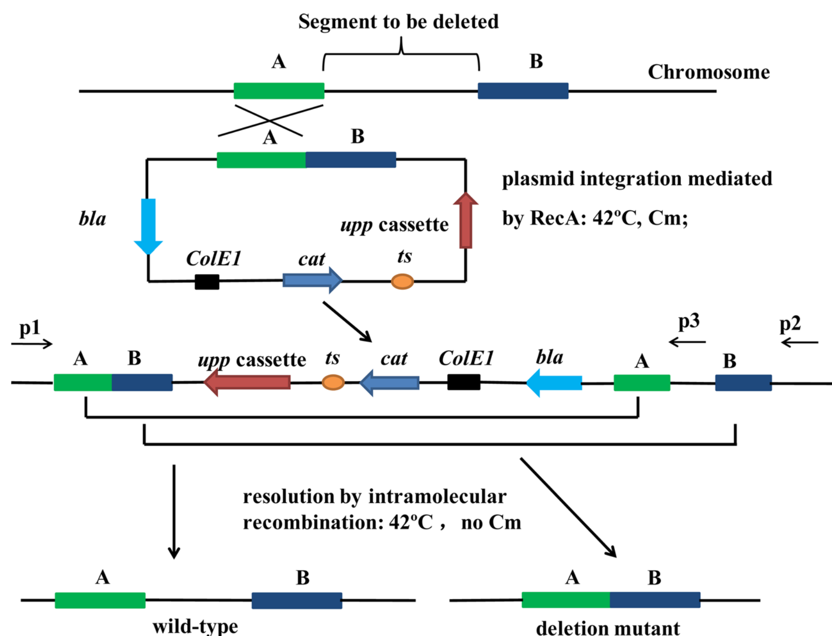

Fig. 2 Scheme of the *upp* and pKSV7-based markerless gene replacement method. *A* and *B* represent homologous arms flanking the segment to be deleted. Single-crossover recombinants were obtained by culturing the transformants at a nonpermissive condition for replication of the temperature-sensitive deletion plasmid involving either *A* or *B*. The cointegrant was then cultured without antibiotic pressure to facilitate intramolecular recombination so as to obtain the desired mutants. Expression of the *upp* cassette in the presence of 5-FU inhibited cells that had not undergone the intramolecular recombination. Recombinants recovered from agar plates with 5-FU either restored to wild-type or gained desired deletion. Chromosomal structures of the mutants were confirmed by PCR and DNA sequencing. Primers for selection are indicated as p1, p2, and p3 in the diagram and explained in detail in the "Results" section

Deletion of the *amyA* gene

Alpha-amylases are induced by starch and hydrolyze its α-1,4 linkages. In *B. amyloliquefaciens* LL3, the gene encoding α-amylase was annotated as *amyA* and assumed to be dispensable in the fermentation process, where sucrose acts as the carbon source. As the first step in demonstrating the efficacy of the gene replacement system, we performed in-frame deletion of the *amyA* gene. Forty random colonies recovered from LB agar containing 5-FU were first tested for their Cm sensitivity by replica plating, and 31 could not grow on LB agar plate supplemented with Cm. PCR performed with primers AmyOUT-F and AmyOUT-R showed that only 2 of the 31 carried an in-frame deletion (Fig. 3b). The two colonies were streaked and grown, and 10 colonies were randomly picked for PCR verification using primers AmyOUT-F and AmyOUT-R again to exclude wild-type strains. The corresponding deletion loci were sequenced, and the obtained sequences showed the successful deletion of the *amyA* gene (data not shown). As a further proof for the deletion of the *amyA* gene, the α-amylase production by LL3 Δ*upp* and LL3 Δ*upp* Δ*amyA* was qualitatively assessed. The absence of a transparent zone around LL3 Δ*upp* Δ*amyA* indicated the absence of α-amylase activity, while a transparent zone appeared around LL3 Δ*upp* because of hydrolysis of starch (Fig. 3c).

Deletion of the partial *bae* cluster

The capacity to delete large DNA fragment of a gene replacement method is preferred for genome reduction, in which a significant amount of chromosomal regions are to be removed. The *bae* cluster is responsible for the synthesis of the secondary metabolite polyketide in LL3 and is identified as a nonessential region (Morimoto et al. 2008). To demonstrate the ability of the gene replacement method to remove long chromosomal segments and accumulate mutations into one strain for genome reduction applications, an approximately 47-kb fragment in the *bae* cluster was targeted in strain LL3 Δ*upp* Δ*amyA*. Primers BaeOUT-F and Bae-M (corresponding to p1 and p3 in Fig. 1, respectively) were used to identify single-crossover recombinants, which were chosen to perform subsequent passage in LB without Cm. The putative double-crossover recombinants recovered from 5-FU plates were tested for their Cm sensitivity. Thirty-two of the 40 colonies were sensitive to Cm, and 8 of the 32 yield a PCR product of approximately 3 kb using primers BaeOUT-F and BaeOUT-R (Fig. 3b). The eight putative deletion-carrying mutants were

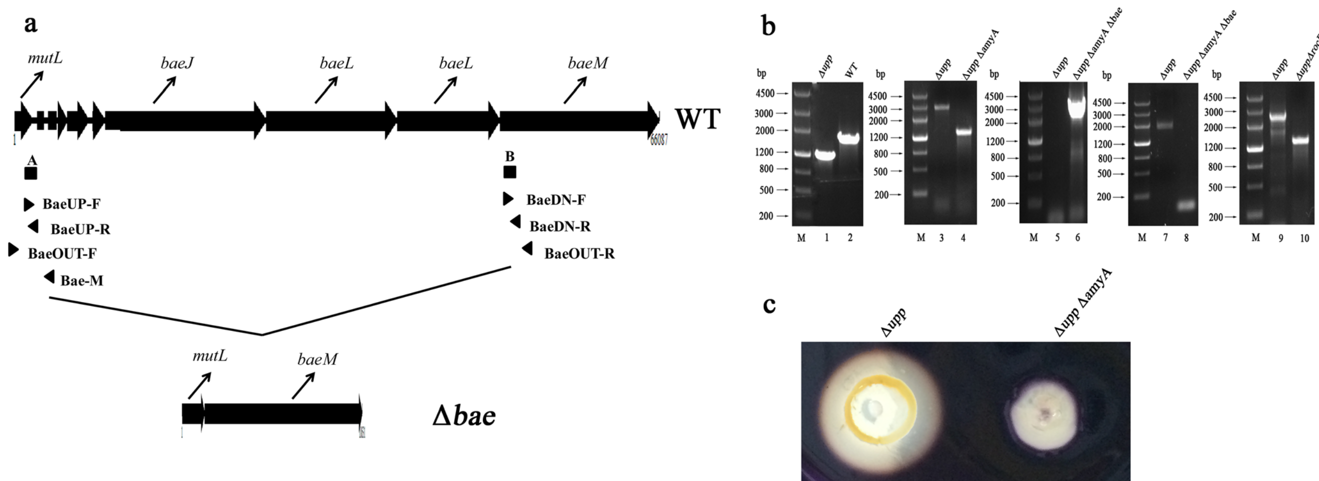

Fig. 3 Confirmation of deletion-carrying mutants by PCR and phenotype. **a** Schematic representation of the genomic loci containing the partial *bae* cluster in *B. amyloliquefaciens* LL3 Δ*upp* and the corresponding genomic deletions in the LL3 Δ*upp* Δ*amy*A Δ*bae*. Open reading frames are indicated as *arrows*. Primers used for PCR screening are shown as *black solid arrows*. Screening for the deletion of other genes is similar. **b** The chromosome structures of deletion-carrying strains were analyzed by agarose gel electrophoresis of PCR products generated (I) with primers UppOUT-F/UppOUT-R (*lanes 1* and *2*); (II) with primers AmyOUT-F/AmyOUT-R (*lanes 3* and *4*); (III) with primers BaeOUT-F/BaeOUT-R (*lanes 5* and *6*) and primers BaeOUT-F/Bae-M (*lanes 7* and *8*); and (IV) with primers RocROUT-F/RocROUT-R. PCR product obtained with primer pairs BaeOUT-F/BaeOUT-R with the genomic DNA of parental strains would be approximately 50 kb, which cannot be generated (*lane 5*). PCR product obtained with the same primer pairs using genomic DNA of Δ*bae* strain as a template is approximately 3 kb (*lane 6*). Primer Bae-M was designed to anneal regions deleted in the Δ*bae* strains. PCR using primer pairs BaeOUT-F/Bae-M with the genomic DNA of the Δ*bae* strain did not result in any fragments (*lane 8*), while PCR with the genomic DNA of the parental strain yields an approximately 2-kb fragment (*lane 7*). *M* indicates DNA marker III, and the size of each band is reported on the *left*. Primer nomenclature is as in Table 2. The genotypes of strains are indicated on the *top of each lane*. Results of *lanes 1–6* and *9–10* showed that the mutated strains selected carried a truncated allele of certain genes or regions, indicating the removal of those fragments from the genome. Results of *lanes 7* and *8* exclude wild-type strains that might be mixed with strains carrying desired mutations. **c** A transparent zone appeared around LL3 because of hydrolysis of the starch in the medium (*left*); by contrast, absence of the transparent zone around LL3 Δ*upp* indicated the absence of α-amylase activity as a result of *amyA* deletion (*right*)

plated on LB agar plates and random colonies were chosen to perform PCR verification. Primers BaeOUT-F and Bae-M were used to exclude wild types that might be mixed with the mutants (Fig. 3b). DNA sequencing of the deletion junction showed that the 3-kb homologous arms had replaced the 47-kb fragment (data not shown).

Deletion of *rocR* promoting γ-PGA production

To construct LL3 Δ*upp* Δ*rocR*, 50 random colonies were picked from LB agar containing 5-FU and 34 showed Cm sensitivity. PCR performed with primers RocROUT-F and RocROUT-R showed that all of the 34 colonies carried an in-frame deletion (Fig. 3b). Two colonies were streaked and grown on LB agar containing 5-FU, and 10 colonies were randomly picked for PCR verification using primers RocROUT-F and RocROUT-R again to exclude wild-type strains. The corresponding deletion loci were sequenced, and the obtained sequences showed the successful deletion of the *rocR* gene (data not shown). The growth of the Δ*rocR* mutant and the Δ*upp* strain were tested in fermentation medium. LL3 Δ*upp* Δ*rocR* presented a shorter period of log phase and a lower biomass compared with LL3 Δ*upp* at the end of the fermentation (Fig. 4b, c). Despite the decreased biomass, the total γ-PGA production (g/L) and the specific γ-PGA production (g/g dry cell weight (DCW)) of LL3 Δ*upp* Δ*rocR* was increased by 1.25- and 1.9-fold, respectively.

It should be noted that complementation of the *rocR* deletion in the form of a plasmid was not successful. The *rocR* gene under the control of its own promoter was ligated in pWH1520, an expression vector for *Bacillus* strains. The *rocR* mutant containing pWH-rocR showed a severe growth defect both in LB and fermentation medium. No apparent growth was observed for the initial 24 h, and OD_{600} reached only 1.1 at 48 h. A very similar growth pattern was also observed in wild-type LL3 harboring pWH-rocR. γ-PGA could not be extracted using the ethanol precipitation method. In contrast, the *rocR* mutant containing pWH1520 showed a similar growth pattern with LL3 Δ*upp* Δ*rocR*. Therefore, we suggested that overexpression of *rocR* may cause disturbance in glutamate metabolism, thus affecting cell growth and γ-PGA production.

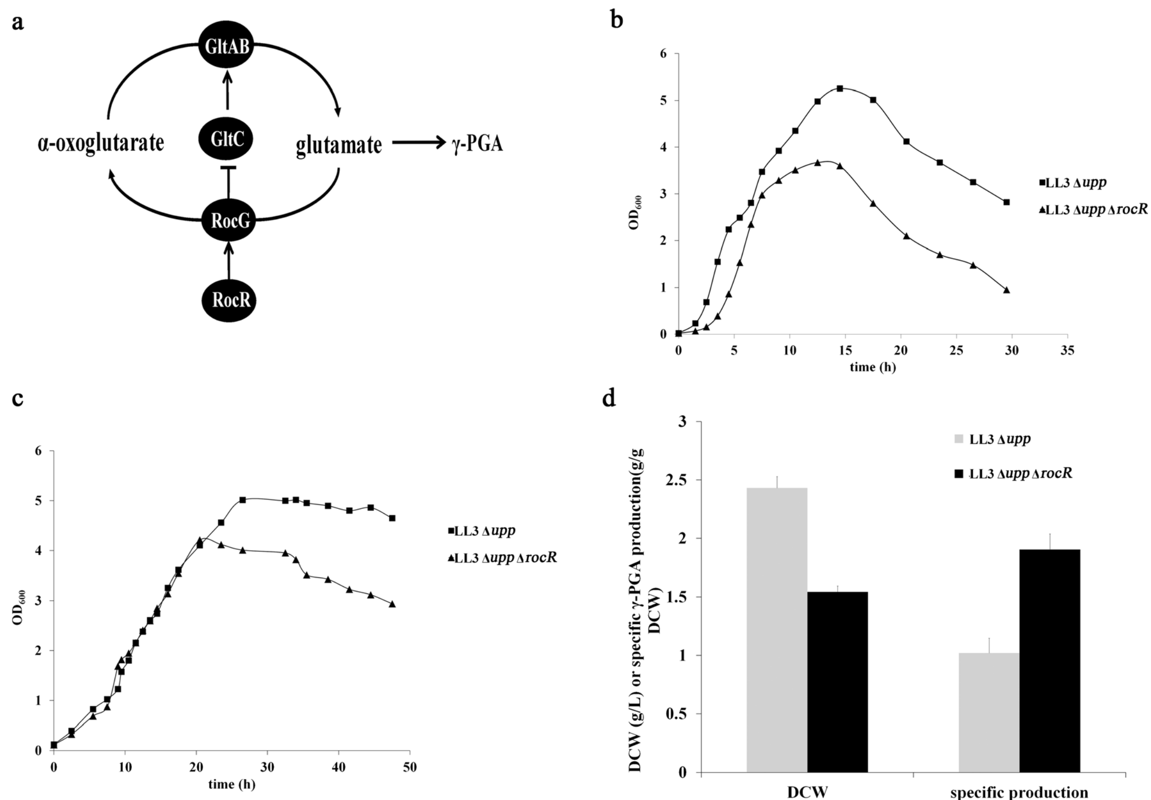

Fig. 4 Effects of RocR on cell growth and γ-PGA production. **a** Speculative glutamate metabolism and its regulation in *Bacillus amyloliquefaciens* LL3, based on reports in *B. subtilis* 168. **b** Growth profiles of Δ*rocR* mutant (*triangle*) and wild-type LL3 Δ*upp* (*square*) in LB medium. **c** Growth profiles of Δ*rocR* mutant (*triangle*) and wild-type LL3 Δ*upp* (*square*) in the fermentation medium. The results presented for growth experiments are the means of three independent replicates, and the standard deviations are below 10 %. **d** γ-PGA production and dry cell weight (DCW) profiles of Δ*rocR* mutant (*black*) and wild-type LL3 Δ*upp* (*gray*). The results presented are the means of biological triplicates. *Error bars* represent standard deviations ($n=3$)

Discussion

When we performed gene insertion or deletion using the pE194ts-replicon plasmid pKSV7 in strain *B. amyloliquefaciens* LL3 without a counterselective marker, the plasmid was not readily excised from the genome. In the case of the insertion of the *vgb* gene in the genome, even with the aid of the kanamycin resistance gene, the desired insertion mutation appeared at a frequency of approximately 0.3 % (Zhang et al. 2013). For the purpose of conducting genome-scale applications, *upp* was introduced to promote the screening of recombinants in which the plasmid has excised. In the selection for double-crossover mutants, even with the aid of *upp*, colonies that had not undergone the second homologous recombinational events were able to grow in the presence of 5-FU. Those false-positive colonies reduced the screening efficiency. In the case of *hisZ* deletion in *B. subtilis*, only 7 % of the 5-FU^R colonies had lost the *upp* cassette (Fabret et al. 2002). The gene replacement method adapted in this study, however, shows a low rate of false positives. All the 5-FU^R colonies in the process of *upp* deletion were positive, and the proportions of positives in the deletion of *amyA*, *bae*, and *rocR* were 77.5, 80, and 68 %, respectively. It is noteworthy that a high plasmid excision rate was reported using the pG$^+$ host-derived plasmid without any counterselectable marker in *B. amyloliquefaciens* and *Lactococcus lactis* (Zakataeva et al. 2010; Biswas et al. 1993). However, using the pE194ts-replicon plasmid pKSV7 in strain LL3, the plasmid excision rate was low and application of the temperature-sensitive plasmid alone is unsuitable for genome-scale applications. Introduction of *upp* greatly facilitated the screening of desired mutants, thus providing an alternative way for markerless genetic manipulation in *B. amyloliquefaciens*. In most of our deletion experiments, 50 colonies recovered from 5-FU plates were enough to obtain recombinant strains carrying the desired mutations. There are several possible explanations for the high rate of true positives. Firstly, it might be caused by the high 5-FU concentration we applied compared with a previous report (Fabret et al. 2002). The cell growth of LL3 was completely inhibited by 1.3 mM of 5-FU on LB agar. Secondly, we postulated that the expression level of the *upp* gene of *B. subtilis* 168 in LL3 is high enough to kill all the plasmid-bearing cells in the presence of 5-FU.

The proportion of deletion-carrying mutants against the colonies that were picked on LB agar with 5-FU for the deletion of the 47-kb partial *bae* cluster was higher than that of the 1.5-kb *amyA* gene. This implied that the deletion efficiency is gene specific, instead of length specific, at least for regions shorter than 47 kb. Our developed method will be of great value for genome reduction applications.

Besides *B. amyloliquefaciens*, the gene replacement system also works for *B. subtilis* (unpublished data). Those results suggest that the method should be applicable to other microorganisms for which plasmids can be introduced. For example, the pE194ts origin of the pKSV7-based deletion plasmid is also compatible with *Staphylococci* strains (Redder and Linder 2012), which suggests that our method may have broader appeal than just *Bacillus*.

However, a few problems remained for this method. First, for every gene deletion, time-consuming cloning procedures have to be performed, taking more than 2 weeks to conduct one round of deletion. In some rare instances, the deletion plasmid could not be constructed because the homologous arms chosen happened to encode toxic substances in *E. coli* cells (data not shown). In these cases, we have to change the region of the homologous arms. However, it should be noted that, to the best of our knowledge, a PCR product-mediated method has not been reported for *B. amyloliquefaciens* strains. Second, the method cannot deal with genes that are difficult to remove. In our attempt to knockout a global regulator AbrB, all of the 200 5-FU^R colonies subjected to PCR verification retained a wild-type genotype (data not shown). One feasible solution is the introduction of a gene encoding a drug-resistant cassette at first and then replacing the antibiotic marker with a markerless copy of the mutant allele.

As a demonstration of the use of our method in improving the production potential of existing producers, we targeted the *rocR* gene, whose deletion was reported to enhance cell growth and enzyme production (Manabe et al. 2011). Glutamate is a central metabolite in all organisms and is synthesized from α-ketoglutarate, an intermediate of the tricarboxylic acid (TCA) cycle. Glutamate serves as the amino group donor for nearly all nitrogen-containing compounds, such as amino acids and nucleotides. In *B. subtilis*, glutamate is exclusively synthesized by the glutamate synthase encoded by the *gltAB* operon, which is activated by a transcription protein, GltC. A regulatory protein, RocG, inhibits GltC. The bifunctional protein RocG has another role as the major glutamate dehydrogenase, exclusively devoted to glutamate degradation and is positively regulated by RocR in *B. subtilis* (Gunka and Commichau 2012). Thus, the deletion of RocR would not only activate glutamate synthesis, but would simultaneously decrease glutamate degradation. It was reported that the deletion of the *rocR* gene led to an increased intracellular glutamate concentration (Manabe et al. 2011, 2013). Our *rocR* deletion mutant produces more PGA compared with wild-type strain; however, it is still unknown whether the increase is directly caused by the absence of RocR or not as the complementation experiment was not successful.

During the screening for Δ*rocR* mutants, all 34 colonies recovered from the LB agar plates supplemented with 5-FU were found to carry an in-frame deletion of the *rocR* gene, which led us to propose that the Δ*rocR* mutant would have growth advantages over its parent strain when grown in LB medium. However, its growth deteriorates, instead of being

enhanced both in LB and fermentation medium (Fig. 4b, c). Therefore, the domination of the Δ*rocR* mutants among the double-crossover mutants may be caused by the genome structure of the co-integrates, which is inclined to resolve into the deletion mutant, instead of the wild type.

The low DCW of the Δ*rocR* strain may be partly caused by the fact that growth and fermentation experiments were performed in shake flasks without pH control, and the pH of the fermentation medium for the Δ*rocR* and Δ*upp* mutant at the end of the fermentation process was 5.8 and 6.5, respectively. Therefore, the pH auxostat fermentation strategy may alleviate the problem (Manabe et al. 2013), restoring cell growth and further improving γ-PGA production, which will require further investigation.

In summary, we have adapted a markerless gene replacement method for the naturally nontransformable *B. amyloliquefaciens* strain LL3 by the combination of a temperature-sensitive plasmid pKSV7 with a counterselectable marker *upp*. We demonstrated this method by performing continuous deletions of *amyA* and the partial *bae* cluster. We also investigated the effects of the transcriptional regulator RocR on γ-PGA production by conducting an in-frame deletion of the *rocR* gene. The rocR mutant shows an increase in specific γ-PGA production, but a decrease in DCW in shake flasks experiments. These results show that our gene replacement method has great potential in the study of genes with unknown functions and in the application of genome reduction for *B. amyloliquefaciens* strains.

Acknowledgments This work was supported by the National Key Basic Research Program of China ("973"-Program) 2012CB725204; the National High Technology Research and Development Program of China ("863"-Program) 2012AA021505; the Natural Science Foundation of China Grant Nos. 31170030, 31300032, and 51073081; and the Project of Tianjin, China (13JCZDJC27800, 13JCYBJC24900, and 13JCQNJC09700). This work was also supported by the Ph.D. Candidate Research Innovation Fund of Nankai University.

Conflict of interest The authors declare that they have no conflict of interest.

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