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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Moderate expression of the transcriptional regulator ALsR enhances acetoin production by *Bacillus subtilis*

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Abstract Acetoin, a major extracellular catabolic product of *Bacillus subtilis* cultured on glucose, is widely used to add flavor to food and also serves as a precursor for chemical synthesis. The biosynthesis of acetoin from pyruvate requires the enzymes α-acetolactate synthase (ALS) and α-acetolactate decarboxylase (ALDC), both of which are encoded by the *alsSD* operon. The transcriptional regulator ALsR is essential for the expression of *alsSD*. Here we focused on enhancing the production of acetoin by *B. subtilis* using different promoters to express ALsR. The expression of reporter genes was much higher under the control of the *Hpa*II promoter than under control of the P_{bdhA} promoter. Although the *Hpa*II promoter highly enhanced transcription of the *alsSD* operon through overexpression of ALsR, the production of acetoin was not significantly increased. In contrast, moderate enhancement of ALsR expression using the P_{bdhA} promoter significantly improved acetoin production. Compared with the wild-type, the enzyme activities of ALS and ALDC in *B. subtilis* harboring P_{bdhA} were increased by approximately twofold, and the molar yield of acetoin from glucose was improved by 62.9 % in shake flask fermentation. In a 5-L fermentor, the engineered *B. subtilis* ultimately yielded 41.5 g/L of acetoin. Based on these results, we conclude that enhanced expression of ALDC and ALS by moderately elevated expression of the transcriptional regulator ALsR could increase acetoin production in recombinant *B. subtilis*.

Keywords *Bacillus subtilis* · Acetoin · AlsR · P_{bdhA} promoter · Moderate regulation

Abbreviations

ALDC	α-Acetolactate decarboxylase
ALS	α-Acetolactate synthase
AlsR	Transcriptional regulator
AR/BDH	Acetoin reductase/2,3-butanediol dehydrogenase
CAT	Chloramphenicol acetyltransferase
GFP	Green fluorescent protein
GRAS	Generally recognized as safe

Introduction

Acetoin (3-hydroxy-2-butanone), a naturally occuring compound in certain fruits, is widely used to add flavor to food and also serves as a precursor in the synthesis of many important compounds. Acetoin plays important physiological roles in microorganisms. Its accumulation is regarded as an energy-storing strategy. It can also neutralize metabolic acidosis [26], and during sugar fermentation, acid pyruvate can be transformed into acetoin to neutralize the acidic environment in *Bacillus subtilis* [11,

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17]. In *Staphylococcus aureus*, the generation of acetoin is also related to cell death [29].

Acetoin can be chemically synthesized from diacetyl or butanol [5, 12]. However, recent attention has focused on developing microbial fermentation methods because they use inexpensive starting materials and the process is much less hazardous [3, 7, 19, 30]. Moreover, microbial production better meets the increasing demand for food safety. Although the production of acetoin has been improved by the screening of more productive bacterial strains or by optimizing the fermentation medium [27], significant amounts of the byproduct 2,3-butanediol are generated [28, 31, 32]. The highest acetoin production ever reported was achieved by *Serratia marcescens*, but the use of pathogens is inappropriate for the industrial production of food additives [25].

Acetoin biosynthesis from pyruvate involves the enzymes α-acetolactate synthase (ALS) and α-acetolactate decarboxylase (ALDC), which are encoded by the *alsSD* operon in *B. subtilis* [20]. Only when the intracellular pyruvate concentration reaches a high level [23, 27] and is accompanied by the expression of ALS does acetoin markedly accumulate [16]. It has been reported that the transcriptional regulator AlsR is essential for the expression of the *alsSD* operon [9] and that the disruption of *alsR* prevents the transcription of *alsSD* [20]. Biswas et al. [2] proposed that activation of the *alsSD* operon is vital for acetoin production.

Bacillus subtilis, which is a generally recognized as safe (GRAS) strain, is able to produce acetoin as its major fermentation product [32]. When fermented with glucose, this microorganism transforms acetoin into 2,3-butanediol in the early stages, while 2,3-butanediol is then reversely transformed into acetoin in the late stages. The interconversion between acetoin and 2,3-butanediol, catalyzed by acetoin reductase/2,3-butanediol dehydrogenase (AR/BDH), prompted us to conduct a series of experiments. The enzyme AR/BDH from *B. subtilis* strain 168 was first expressed, purified, and characterized by us to better understand the reversible reactions. Since *B. subtilis* produces acetoin or 2,3-butanediol as its major product depending on the different fermentation stage, the promoter function of the *bdhA* gene (encoding AR/BDH) was investigated to determine whether the expression of AR/BDH is associated with the physiological status of the cells. In addition, we attempted to overexpress ALS and ALDC to enhance acetoin production in our laboratory. We succeeded in increasing the activity of these enzymes by more than 100-fold; however, acetoin production was not significantly enhanced. The overexpression of ALS and ALDC markedly inhibited cell growth, possibly by competing with other metabolic pathways, such as glycolysis. To further increase the yields of acetoin, we reasoned that this could be accomplished by controlling the expression of ALS and ALDC using ALsR, the regulator of the *alsSD* operon.

In the study reported here, the effects of using either strong (*Hpa*II) or weak (P_{bdhA}) promoters to drive ALsR expression were compared. Our results demonstrate that the yield of acetoin was the highest using the weaker promoter.

Materials and methods

Materials

The PCR reagents, T_4 DNA ligase, restriction enzymes, RNAiso Plus, PrimeScript RT Reagent kit, and SYBR *Premix Ex Taq*™ II were purchased from TaKaRa (Dalian, China). The Mini Plasmid Rapid Isolation kit and Mini DNA Rapid Purification kit were purchased from Sangon Biotech (Shanghai, China). Experiments were performed in accordance with the manufacturers' instructions.

Strains, plasmids, and primers

The bacterial strains, plasmids, and primers used in this work are listed in Table 1.

Culture conditions

Bacillus subtilis and *Escherichia coli* were cultured in Luria–Bertani (LB) medium at 37 °C on a rotary shaker at 160 rpm. When necessary, the LB medium was supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin. For acetoin fermentation, the cells were inoculated into 10 mL of LB medium and cultivated for 7 h on a rotary shaker at 160 rpm, and then 2 mL of culture was transferred into 50 mL of seed medium (LB with 40 g/L glucose) for preparing a seed culture. After 10 h, 2.5 mL of the seed culture (OD_{600} = 5–6) was inoculated into 50 mL of fermentation medium (in g/L; glucose, 100; beef extract, 5; corn steep liquor,; urea 2; pH 6.8) for 144 h. The cell-free supernatants were prepared at 24-h intervals by centrifuging the cultures at 8,000 rpm for 10 min followed immediately by storage at −20 °C. When cells were cultured in a 5-L fermentor (Biotech, Shanghai, China), the agitation speed and pH were 300 rpm and 6.8, respectively. The initial glucose concentration was 120 g/L.

PCR amplification of DNA fragments

Polymerase chain reaction was performed using a Peltier Thermal Cycler (Bio-Rad, Hercules, CA). PCR mixtures (50 μL) contained 1 ng of template, 200 μM dNTP, 20 μM

Table 1 Bacterial strains, plasmids, and primers

Strains/plasmids/primers	Characteristics	Source
Strains		
Escherichia coli		
JM109	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsd R17*(r_k^- m_k^+)*supE44*	Invitrogen
Bacillus subtilis		
JNA 3-10	Wild-type, produces acetoin and 2,3-butanediol	Laboratory stock
BS168	*trpC2*	Laboratory stock
BS-G1	*B. subtilis* 168 containing pMA5-G1 (Km^R)	This study
BS-G2	*B. subtilis* 168 containing pMA5-G2 (Km^R)	This study
BS-PA	*B. subtilis* 168 containing pMA5-PA (Km^R)	This study
BS-PAG	*B. subtilis* 168 containing pMA5-PAG (Km^R)	This study
BS-C	*B. subtilis* 168 containing pMA5-C (Km^R)	This study
BS-PAC	*B. subtilis* 168 containing pMA5-PAC (Km^R)	This study
BS-R	*B. subtilis* 168 containing pMA5-R (Km^R)	This study
BS-PAR	*B. subtilis* 168 containing pMA5-PAR (Km^R)	This study
BS-R-PAR	*B. subtilis* 168 containing pMA5-R-PAR (Km^R)	This study
BS-RG	*B. subtilis* 168 containing pMA5-RG (Km^R)	This study
BS-PARG	*B. subtilis* 168 containing pMA5-RARG (Km^R)	This study
Plasmids		
pCAMBIA1302	Containing *gfp*	Laboratory stock
pACYCDuet™-1	Containing *cat*	Novagen
pMA5	*Hpa*II, colE1, *repB*, replicates in *E. Coli* (Amp^R) or *B. Subtilis* (Km^R)	Laboratory stock
pMA5-G (1, 2)	pMA5 containing *gfp* (P1 and P2, P3 and P4)	This study
pMA5-PA	pMA5 containing P_{bdhA}	This study
pMA5-PAG	pMA5-PA containing *gfp*	This study
pMA5-C	pMA5 containing *cat*	This study
pMA5-PAC	pMA5-PA containing *cat*	This study
pMA5-R	pMA5 containing *alsR*	This study
pMA5-PAR	pMA-PA containing *alsR*	This study
pMA5-R-PAR	pMA5 containing *alsR* and P_{bdhA}-*alsR*	This study
pMA5-RG	pMA5 containing *alsR-gfp*	This study
pMA5-RARG	pMA-PA containing *alsR-gfp*	This study
Primers 5′–3′		
P1	ACCG***GAATTC***TTCGTCCCCCTGTTTGTT (*Eco*RI)	
P2	ACCG***GATATC***GGATTACCACTCCTATAACT (*Eco*RV)	
P3	ACCG***GGATCC***ATGGTAGATCTGACTAG (*Bam*HI)	
P4	ACCCG***ACGCGT***CCCGATCTAGTAACATAG (*Mlu*I)	
P5	ACCG***GATATC***ATGGTAGATCTGACTAG (*Eco*RV)	
P6	ACCCG***AAGCTT***CCCGATCTAGTAACATAG (*Hin*dIII)	
P7	ACCG***GGATCC***TTCGAATTTCTGCCATTCATC (*Bam*HI)	
P8	ACCCG***ACGCGT***GCGGTGCTTTTGCCGTTACG (*Mlu*I)	
P9	ACCG***GATATC***TTCGAATTTCTGCCATTCATC (*Eco*RV)	
P10	ACCCG***AAGCTT***GCGGTGCTTTTGCCGTTACG (*Hin*dIII)	
P11	ACCG***CATATG***ATGGAGCTTCGCCA (*Nde*I)	
P12	ACCG***GGATCC***TCATGTACCTGCATC (*Bam*HI)	
P13	ACCG***GATATC***ATGGAGCTTCGCCA (*Eco*RV)	
P14	ACCG***AAGCTT***TCATGTACCTGCATC (*Hin*dIII)	
P15	ACCG***GGATCC***TGTACCTGCATCACTC (*Bam*HI)	
P16	GCCCAGGTAAGGTTCTTC	

Table 1 continued

Strains/plasmids/primers	Characteristics	Source
P17	GGTGTAGCGGTGAAATGC	
P18	GACGGCGGTTTCTTATTCTC	
P19	ATGAACGCTGAAGGTCCTG	
P20	AATCGCCGTTTCAGGCTATC	
P21	CAACTGAAGGAAGCCCTGAA	

Amp^R Ampicillin resistant, Km^R kanamycin resistant

The highlighted text in the primer sequences represent restriction enzyme sites

primers, and 1 Ut Ex *Taq* DNA polymerase. The PCR amplification conditions were one cycle of 95 °C for 300 s, followed by 35 cycles of denaturation at 95 °C for 50 s, annealing at 56 °C for 90 s, and extension at 72 °C for 90 s, and terminating with a 10-min extension at 72 °C. The amplicons were separated by electrophoresis in a 1.0 % agarose gel. The desired bands were excised and gel-purified.

Construction of recombinant plasmids

The construction of recombinant plasmids is shown in Fig. 1. The promoter P_{bdhA} was amplified using primers P1 and P2 and *B. subtilis* JNA 3-10 DNA as template. The plasmid pMA5-PA was constructed by inserting P_{bdhA} between the *Eco*RI and *Eco*RV sites of pMA5 (Fig. 1a). A green fluorescent protein (GFP) expression cassette was amplified by PCR using primers P3/P4 and P5/P6 from the plasmid pCAMBIA1302. The two purified PCR products were double-digested with *Bam*HI/*Mlu*I or *Eco*RV/*Hin*dIII, respectively, and then ligated to the corresponding sites of pMA5 to generate pMA5-G1 and pMA5-G2. To construct pMA5-PAG, pMA5-PA was digested with *Eco*RV and *Hin*dIII as well as pMA5-G2 which released a short fragment of *gfp*. Linearized pMA5-PA was ligated to this *gfp* fragment (Fig. 1b).

The *cat* gene encoding chloramphenicol acetyltransferase (CAT) was amplified from pACYCDuet™-1 using primer pairs P7/P8 or P9/P10. Plasmids pMA5-C and pMA5-PAC were constructed in the same manner as pMA5-G1 and pMA5-PAG (Fig. 1c).

The *alsR* gene encoding the transcriptional regulator ALsR was amplified using primer pairs P11/P12 and P13/P14, and *B. subtilis* JNA 3-10 DNA was used as the template. The plasmids pMA5-R and pMA5-PAR were constructed in the same manner as pMA5-C and pMA5-PAC. The plasmid pMA5-R-PAR was constructed by inserting *alsR* into pMA5-PAR between the *Bam*HI and *Mlu*I sites (Fig. 1d).

The plasmids pMA5-RG and pMA5-PARG were constructed to express an ALsR-GFP fusion (Fig. 1d). The *alsR* gene was amplified using primers P11 and P15 and then inserted into pMA5-G1, generating pMA5-RG. P13 and P6 were used to amplify the *alsR-gfp* fusion gene from pMA5-RG, and the fragment was then inserted into pMA5-PA, generating pMA5-PARG.

Transformation and selection of recombinant strains

The ligated DNAs were used to transform *E. coli* JM109. Positive colonies were selected on agar plates containing ampicillin, and the plasmids were confirmed using restriction enzyme analysis and DNA sequencing. The recombinant plasmids were then used to transform *B. subtilis* 168 [6]. The transformants were screened for their ability to grow on LB agar plates containing kanamycin.

Preparation of crude enzymes and determination of specific activities of ALS, ALDC, AR/BDH and CAT

Cells were cultured and centrifuged at 8,000 rpm for 10 min and the supernatants were then stored at −20 °C. For determining AR/BDH activity [18], the cell pellets were washed three times with 0.1 M potassium phosphate buffer (pH 7.0) and then suspended in 0.1 M potassium phosphate buffer (pH 6.5) containing 0.1 mM β-mercaptoethanol and 2 μg/mL phenylmethylsulfonyl fluoride. For other enzymes, the cell pellets were washed three times with wash buffer (0.2 mM NaH_2PO_4, 2.2 mM Na_2HPO_4, and 8.5 mM NaCl; pH 7.4) and then suspended in this buffer. Cells were disrupted using a sonicator (SONICS, Newtown, CT) for 20 min with chilling. Cell debris was removed by centrifugation at 12,000 rpm for 30 min at 4 °C. The ALS, ALDC [1, 13], and CAT [22] assays were performed according to published procedures.

Preparation of RNA and cDNA synthesis

Total RNA was isolated from *B. subtilis* using RNAiso Plus reagent according to the manufacturer's instructions

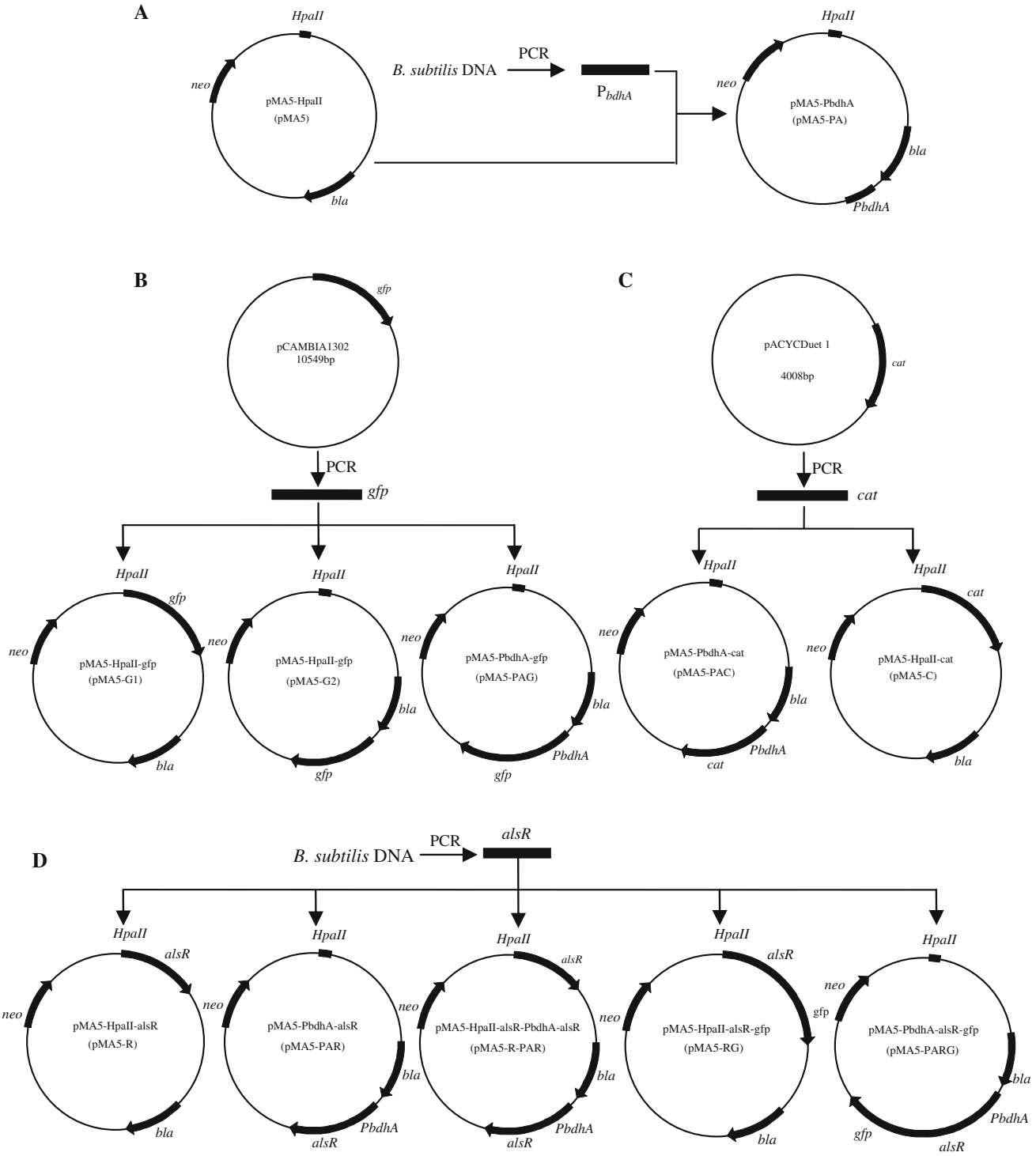

Fig. 1 Construction of the recombinant plasmids. **a** pMA5-PA construction in which the P_{bdhA} promoter was amplified from *Bacillus subtilis* strain JNA 3-10, **b** pMA5-G1, pMA5-G2, and pMA5-PAG construction in which the green fluorescent protein gene (*gfp*) was amplified from plasmid pCAMBIA1302, **c** pMA5-C and pMA5-PAC construction in which the chloramphenicol acetyltransferase gene (*cat*) was amplified from plasmid pACYCDuet^{TM}-1, **d** pMA5-R, pMA5-PAR, pMA5-R-PAR, pMA5-RG, and pMA5-PARG construction in which the transcriptional regulator of the α-acetolactate synthase gene (*alsR*) was amplified from *B. subtilis* JNA 3-10 and the termination codon gene of *alsR* was deleted in pMA5-RG and pMA5-PARG

and purified as previously described [8]. RNA was dissolved in water treated with diethyl pyrocarbonate and analyzed using 1 % agarose gel electrophoresis. A standard PCR assay was used to test for the presence of contaminating genomic DNA. The RNA preparation was judged suitable for subsequent analyses when the ratio of absorbance at 260/280 nm was between 1.9 and 2.1 and the 260/230 nm ratio was greater than 2.0. RNAs were converted to cDNAs using a PrimeScript RT reagent kit containing 2 μg RNA, 10 μL PrimeScript Buffer, 2.5 μL PrimeScript RT Enzyme Mix I, 2.5 μL oligo dT primer, and 2.5 μL random hexamers; the final volume was adjusted to 50 μL using RNase-free dH_2O. The reaction mixture was incubated at first 42 °C for 15 min and then at 85 °C for 5 s. All cDNA samples were analyzed using PCR before further analysis.

Primer design for real-time PCR

The sequences of the 16S rDNA gene, *alsS*, and *alsD* were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank). Specific primers (P16–P21) were designed using Primer 5 software to obtain amplicons of <270 bp, melting points of 58 ± 0.5 °C, and a GC content between 40 and 60 %.

Real-time PCR

Real-time PCR was performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System using the SYBR *Premix Ex Taq*™ II. The PCR mixture was prepared containing 1 μL of cDNA (1:10 diluted), 10 μL of SYBR *Premix Ex Taq*™ II, 1 μL of each primer (10 μM), and dH_2O, up to a total volume of 20 μL. Thermal cycling conditions were on cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 56 °C for 15 s and 72 °C for 20 s. Melting curves were analyzed over the range 65 to 95 °C to ensure that the resulting PCR products did not represent primer-dimers or nonspecific products formed during the PCR. Negative controls without template were also included to detect any spurious signals of DNA contamination. All reactions were performed in triplicate biological replicates.

Analytical methods

Biomass was measured spectrophotometrically at 600 nm (UNICO UV-2000, USA) after the cultures were diluted with water to obtain linear readings. Residual glucose was detected using a SBA-40C biological sensing analyzer (SBA, China). Fermentation products present in cell-free supernatants were monitored periodically. The concentrations of acetoin and 2,3-butanediol were monitored using gas chromatography [Dong Xi GC2000A, FID detector; 2 m × 5 (diameter)-mm packed column, N_2 flow rate of 50 mL/min, detector temperature 240 °C, and a column temperature of 220 °C]. All assays were performed in duplicate or triplicate.

Results

Functional comparison of the promoters *Hpa*II and P_{bdhA} in *B. subtilis*

The plasmids pMA5-G1, pMA5-G2, pMA5-PA, and pMA5-PAG were constructed as described in the Materials and methods (Fig. 1a, b). The transcriptional activities of the promoters *Hpa*II and P_{bdhA} were compared by determining the levels of the GFP reporter expression. The locations of reporter genes and promoters are shown in Fig. 1. Plasmid pMA5-G2 was used as a control of pMA5-PAG to eliminate the interference of *Hpa*II on GFP expression.

B. subtilis strains transformed by these recombinant plasmids are designated as BS-G1, BS-G2, BS-PA, and BS-PAG, respectively. The levels of GFP expressed by strains BS-G1 (Fig. 2a), BS-G2 (Fig. 2b), and BS-PAG (Fig. 2c) were determined using NIS viewer software (Nikon, Tokyo, Japan). BS-G1 cultures emitted much more intense fluorescence than BS-PAG cultures, suggesting that GFP was expressed more efficiently by BS-G1 than by BS-PAG. The fluorescence of BS-G2 was undetectable, indicating that the presence of *Hpa*II upstream did not contribute to GFP expression in pMA5-PAG. These results demonstrated that *Hpa*II was a more efficient promoter than P_{bdhA} for driving GFP expression.

Analysis of the transcriptional efficiencies of *Hpa*II and P_{bdhA}

The transcriptional efficiencies of *Hpa*II and P_{bdhA} were quantitated by determining CAT activity. Plasmids pMA5-C and pMA5-PAC were constructed by replacing *gfp* with *cat* (Fig. 1c). The CAT activities of the corresponding recombinant strains BS-C and BS-PAC were 825 and 15 mU/mL, respectively. CAT activity expressed by BS-C was more than 50-fold higher than that of BS-PAC. The results suggested that the transcriptional efficiency of the *Hpa*II promoter was greater than that of P_{bdhA}.

Analysis of ALS and ALDC expression controlled by *Hpa*II and P_{bdhA} promoters

The transcriptional regulator ALsR controls the expression of the *alsSD* (*alsS* and *alsD*) operon [20]. Plasmids pMA5

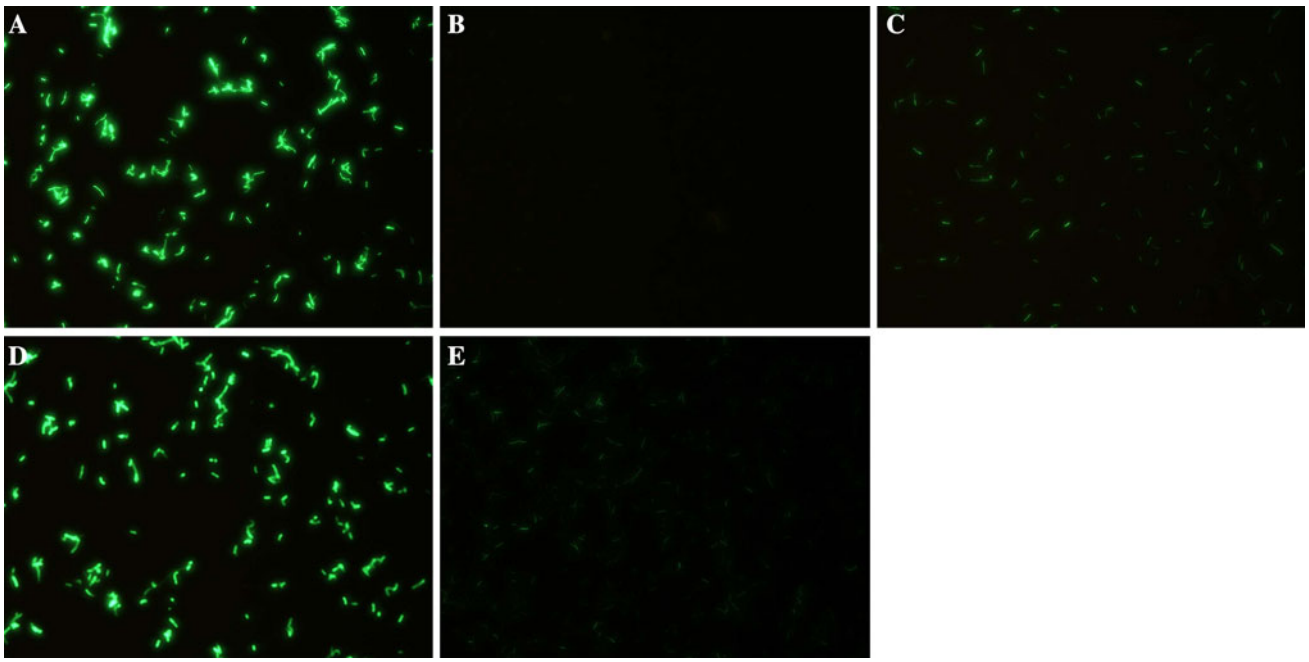

Fig. 2 The detection of GFP using a fluorescent electronic microscope. **a–e** Strength of fluorescence of strains BS-G1 (**a**), BS-G2 (**b**), BS-PAG (**c**), BS-RG (**d**), and BS-PARG (**e**)

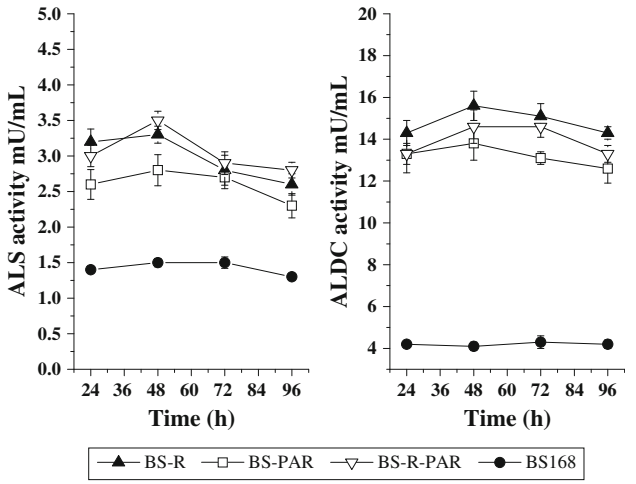

Fig. 3 Assays of ALS and α-acetolactate decarboxylase (*ALDC*) activities in shake flask when fermented with glucose. Assays were carried out a 24-h intervals

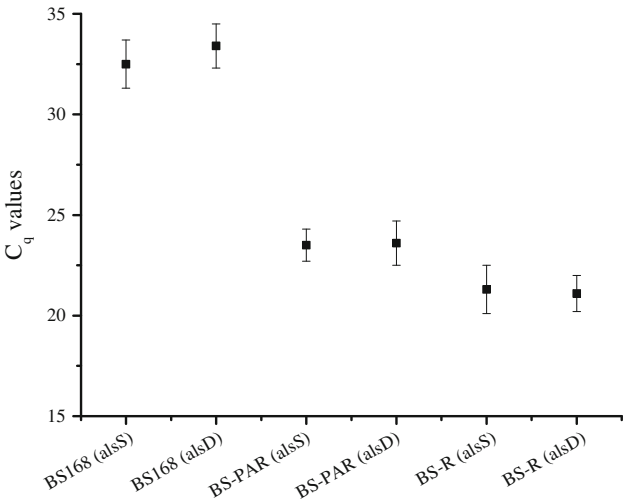

Fig. 4 Relative transcriptional levels of the *alsS* and *alsD* genes in strains BS168, BS-R, and BS-PAR. Values are given as quantification cycle (C_q) values. *Bars* indicate standard deviations (SD)

and pMA5-PA were employed to construct vectors (pMA5-R, pMA5-PAR, and pMA5-R-PAR) that express ALsR (Fig. 1d). The transformants generated were designated as BS-R, BS-PAR, and BS-R-PAR, respectively. The wild-type (BS168) was used as a control. Levels of AlsR were monitored by monitoring its expression as a GFP–fusion protein. The fluorescence in the recombinant strain BS-RG was stronger than that in strain BS-PARG (Fig. 2d, e), suggesting that the expression level of ALsR was higher when controlled by *Hpa*II.

The activities of ALS and ALDC in strains BS168, BS-R, BS-PAR, and BS-R-PAR were determined in fermentation batches (Fig. 3). The highest activities of ALS and ALDC were detected at approximately 48 h in all of the strains except BS168. The total activities of ALS and ALDC were 18.9, 16.6, and 18.1 mU/mL in strains BS-R, BS-PAR, and BS-R-PAR, respectively, and were approximately threefold higher than that in BS168. ALS and ALDC activities were higher in strains BS-R and BS-R-PAR. These results indicated that the expression of ALsR

resulted in more efficient expression of *alsSD* under the control of *Hpa*II than P_{bdhA}.

To assess whether there were differences in the transcriptional levels of *alsSD* between the wild-type and the transformants, real-time PCR was used to determine their quantification cycle (C_q) values (Fig. 4). The C_q values were the highest for BS168 (32.5 and 33.4) and the lowest for BS-R (21.1 and 21.3). Therefore, overexpression of ALsR led to a higher transcription of the *alsSD* operon. The data are consistent with the enzyme activities of ALS and ALDC described above.

Optimum promoter for acetoin production

Yields of acetoin and 2,3-butanediol were determined in *B. subtilis* strains expressing ALsR under the control of either *Hpa*II or P_{bdhA}. To better define the carbon flux, we also determined the levels of major byproducts (ethanol, acetate, and lactate) of acetoin fermentation. The results are summarized in Table 2 and Fig. 5. Strain BS168 had the highest rates of glucose consumption and growth, but also the lowest acetoin production. Although strain BS-R had the highest ALS and ALDC activities, its production of acetoin was only 12.5 % higher than that of BS168. Acetoin and 2,3-butanediol were produced at the highest levels by BS-PAR, and the molar yield of acetoin from glucose was improved to approximately 62.9 %. Unexpectedly, the yields of 2,3-butanediol were higher in all recombinants compared with the wild-type.

We also determined whether AR expression was enhanced by ALsR. The levels of AR activity in all recombinants and BS168 were approximately 0.16 U/mL, suggesting that ALsR had no effect on AR expression. The metabolic flow from pyruvate to acetoin was competitively enhanced by the expression of ALsR because the yields of other metabolites, such as ethanol, acetate, and lactate, were reduced (Table 2).

The curves of glucose fermentation by BS-PAR in a 5-L fermentor are shown in Fig. 6. All of the glucose (120 g/L) was consumed within 96 h, and BS-PAR produced approximately 41.5 g/L acetoin and 6.3 g/L 2,3-butanediol, respectively. The molar yield of acetoin from glucose was approximately 70.7 %, while the total molar yield of acetoin and 2,3-butanediol from glucose was approximately 81.4 %. Therefore, acetoin production was markedly enhanced using a *B. subtilis* strain engineered to express relatively moderate levels of ALsR.

Discussion

The ALsR is essential for the expression of the *alsSD* operon, which directs the synthesis of acetoin by *B. subtilis* [9]. In our study, the transcriptional levels of *alsSD* and the enzymatic activities of ALS and ALDC in recombinants were higher than those of the wild-type BS168, suggesting that ALsR expression enhanced *alsSD* expression. Further, acetoin synthesis by *B. subtilis* was enhanced by regulating ALsR expression using either of the two promoters. Although ALsR expression was higher using the stronger promoter (*Hpa*II) than the moderate promoter (P_{bdhA}), acetoin production did not correlate with the expression levels of ALsR. Compared with the wild-type, acetoin production was improved by 12.5 and 32.8 % under the control of *Hpa*II and P_{bdhA}, respectively. The molar yield of acetoin from glucose was improved to 62.9 % using P_{bdhA}.

Wild-type *B. subtilis* had a faster growth rate than the recombinant strains, suggesting that ALsR expression in the latter inhibited cell growth. A coenzyme (NADH or NAD^+) is required for acetoin biosynthesis [18]. The change in the ratio of NADH/NAD^+ would then affect the growth of cells harboring the expression vectors [21]. This results suggests that the overexpression of ALsR in our system likely enhanced acetoin metabolism at the expense of metabolic factors required for growth.

In wild-type cultures, 2,3-butanediol accumulated quickly during the logarithmic phase and was converted to acetoin when growth was inhibited. By contrast, in the recombinant strains, 2,3-butanediol accumulated gradually and was not detectably converted to acetoin in the later

Table 2 Major products of *B. subtilis* in acetoin fermentation

Strains	Acetoin (g/L)	2,3-Butanediol (g/L)	Alcohol (g/L)	Acetate (g/L)	Lactate (g/L)	Molar yield of acetoin (%)	Molar yield of acetoin and 2,3-butanediol (%)
BS168	23.2 ± 1.1	6.7 ± 0.7	1.2 ± 0.12	2.3 ± 0.21	0.33 ± 0.03	47.4	61.1
BS-R	26.1 ± 1.4	9.3 ± 0.5	0.9 ± 0.14	1.6 ± 0.15	0.26 ± 0.04	53.3	72.4
BS-R-PAR	26.2 ± 1.7	9.2 ± 0.7	0.8 ± 0.13	1.6 ± 0.15	0.28 ± 0.03	53.5	72.4
BS-PAR	30.8 ± 1.5	10.0 ± 0.8	0.8 ± 0.11	1.7 ± 0.13	0.25 ± 0.02	62.9	83.4

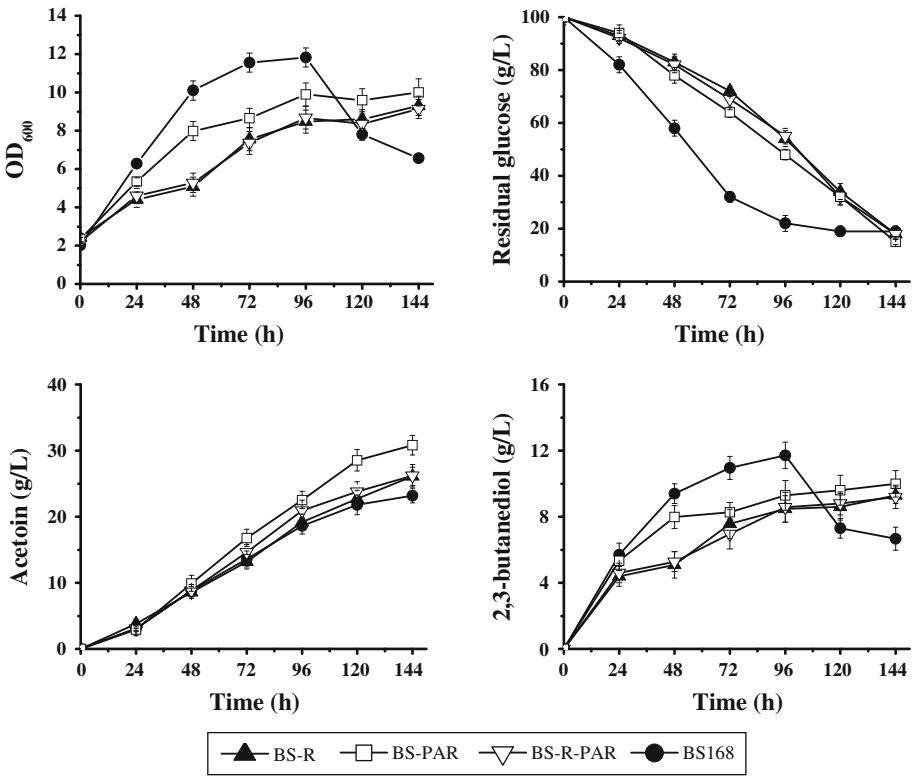

Fig. 5 *Curves* of the cell-growth, residual glucose, acetoin yield and 2,3-butanediol yield in shake flask fermentation by strains *BS168*, *BS-R*, *BS-R-PAR*, and *BS-PAR*

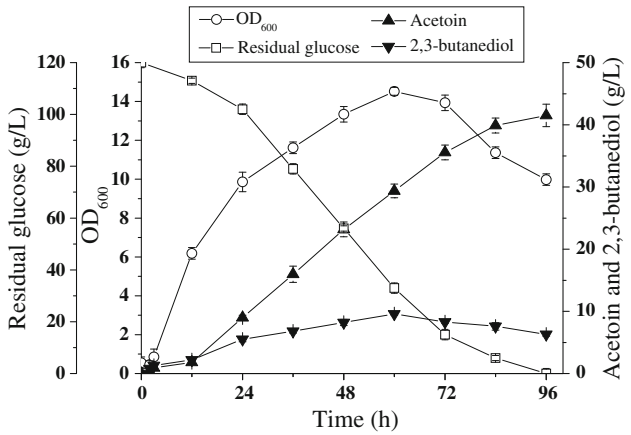

Fig. 6 Fermentation *curves* of strain BS-PAR in a 5-L fermentor

stages of growth, likely due to the intracellular NADH/NAD^+ ratios under different physiological conditions. Cofactor engineering is now an important approach for enhancing acetoin or 2,3-butanediol production [10, 24]. Ji et al. [14] showed that decreasing the intracellular NADH/NAD^+ ratios improved acetoin production, with the accumulation of 2,3-butanediol nearly unaltered.

Another possible cause of increased 2,3-butanediol production by the recombinants involves enhanced AR expression through the regulation of ALsR. However, this conclusion is in disagreement with our findings that AR activities in the recombinant strains were not significantly different from that of BS168. This result is consistent with those of the previous studies demonstrating that ALsR is required only for the transcription of the *alsSD* operon [9, 20]. Therefore, ALsR would not enhance the expression of AR.

To better understand carbon flux in *B. subtilis*, we determined the levels of ethanol, acetate, and lactate. However, the data were complicated and difficult to interpret, and further studies with other acidic metabolites are required. In cultures carried out in a 5-L fermentor, pH 6.8 was maintained, and the yield of acetoin was improved to approximately 41.5 g/L, which is the highest yield by an engineered *B. subtilis* strain.

In summary, the moderate-regulation strategy described here was successful for enhancing acetoin synthesis by *B. subtilis*. However, further modifications, including prevention of acetoin degradation and/or competition with other biosynthetic pathways for its precursors, are required. Other aspects for optimization, such as varying the composition of the culture medium and controlling pH [4, 15] are currently being explored in our laboratory.

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