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ORIGINAL PAPER

Improving the *Clostridium acetobutylicum* butanol fermentation by engineering the strain for co-production of riboflavin

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Abstract Solvent-producing clostridia are well known for their capacity to use a wide variety of renewable biomass and agricultural waste materials for biobutanol production. To investigate the possibility of co-production of a high value chemical during biobutanol production, the *Clostridium acetobutylicum* riboflavin operon *ribGBAH* was over-expressed in *C. acetobutylicum* on *Escherichia coli–Clostridium* shuttle vector pJIR750. Constructs that either maintained the original *C. acetobutylicum* translational start codon or modified the start codons of *ribG* and *ribB* from TTG to ATG were designed. Riboflavin was successfully produced in both *E. coli* and *C. acetobutylicum* using these plasmids, and riboflavin could accumulate up to 27 mg/l in *Clostridium* culture. Furthermore, the *C. acetobutylicum* purine pathway was modified by over-expression of the *Clostridium purF* gene, which encodes the enzyme PRPP amidotransferase. The function of the plasmid pJaF bearing *C. acetobutylicum purF* was verified by its ability to complement an *E. coli purF* mutation. However, co-production of riboflavin with biobutanol by use of the *purF* over-expression plasmid was not improved under the experimental conditions examined. Further rational mutation of the *purF* gene was conducted by replacement of amino acid codons D302 V and K325Q to make it similar to the feedback-resistant enzymes of other species. However, the co-expression of *ribGBAH* and *purFC* in *C. acetobutylicum* also did not improve riboflavin production. By buffering the culture pH, *C. acetobutylicum* ATCC 824(pJpGN) could accumulate more than 70 mg/l riboflavin while producing 190 mM butanol in static cultures. Riboflavin production was shown to exert no effect on solvent production at these levels.

Keywords *Clostridium acetobutylicum* · Biofuel · Riboflavin · Co-production · Purine pathway

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Introduction

Increasing concerns worldwide over the cost of energy, security of energy sources and global warming in association with liquid fossil fuels have resulted in attention being focused on biofuels as substitutes for petroleum-derived transportation fuels. Among the varieties of biofuels currently available, bioethanol as a partial replacement gasoline has gained the attention of commercial concerns during the last few years, with the result that much progress has been made in terms of improved strain development, fermentation processes for cellulosic biomass and separation techniques [1, 15]. Biobutanol is a superb biofuel with many advantages over bioethanol, such as higher energy content, higher blending rate with gasoline without engine modification, convenient distribution using current pipeline infrastructure and better auto emission performance [10].

Butanol fermentation, commonly known as the ABE (acetone–butanol–ethanol) fermentation, was one of the largest biotechnological industry process developed, second only to ethanol fermentation [24]. However, the ABE fermentation industry was gradually phased out in most countries during the 1950s due to the fact that the solvent could only accumulate to less than 2% in the broth, which made it unable to compete with the booming petrochemical industry. In recent years, interest in the ABE fermentation

has resurfaced because of the new global support for the exploitation of biomass as a sustainable source of energy and the great fluctuation in the petro-oil price. However, two major bottlenecks still hamper the economics of the ABE fermentation: (1) the high cost of the substrates and (2) the toxic nature of butanol, which prohibits high product accumulation in the fermentation broth [7]. To overcome the economic bottlenecks for the efficient biological production of biobutanol, various research areas have been addressed with the aim of improving the biobutanol production process and strengthening its competitive status versus the petrochemical industry. During recent years, efforts have been made in the use of low-cost and sustainable raw materials, incorporation of in situ butanol recovery and other separation technologies. Continuous culture platforms have also been investigated [13]. The genetic improvement of the butanol-producing strain has become a highly researched topic since genome sequence data have become available for several solventogenic clostridia strains and genetic transformation systems have been developed. Several recombinant strains have been constructed in our laboratory for better performance of *Clostridium acetobutylicum* ATCC 824 strain [17, 34, 39].

Flavocoenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are derived from riboflavin (vitamin B_2), are absolutely required in all cells as cofactors for a variety of redox enzymes. Riboflavin is biosynthesized by plants and numerous microorganisms—but not by vertebrates. Thus, this essential vitamin has to be provided in the diet. The biosynthesis pathway of riboflavin has been reviewed comprehensively [3]. The biological production of riboflavin by means of a variety of microbial fermentations has been possible since the 1940s, with the fermentative production of riboflavin by various clostridia being the first commercial-scale biological production of riboflavin [43]. To date, different bacteria, yeast, fungi and plants have been studied for their capability for riboflavin production [9, 20]. Since the late 1990s, genetic recombinant techniques have been used to improve riboflavin production.

Recombinant *Bacillus subtilis* is one of the best-known microorganisms for riboflavin production during fed batch fermentation, with riboflavin accumulating up to 15 g/l in a bioreactor [43]. The strategy for improving riboflavin production by *B. subtilis* has been to screen feedback inhibition-resistant mutant strains and increase the riboflavin operon expression by using strong promoters and increasing the copy number. The riboflavin synthesis system in *B. subtilis* is well studied. The *rib* operon encodes a pyrimidine deaminase/reductase (*ribG*), an α-subunit of riboflavin synthase (riboflavin synthase, *ribB*), a GTP cyclohydrolase/3,4-dihydroxy 2-butanone 4-phosphate (3,4-DHBP) synthase (*ribA*), and the ß-subunit of riboflavin synthase (lumazine synthase, *ribH*). The dual function gene *ribA* plays an important function in riboflavin over-production in *B. subtilis* [19]. The *rib* operon has been characterized in many organisms; however, the nomenclature of genes varies among species. The original nomenclature used in *B. subtilis* is adopted in this paper.

In this paper we demonstrate the possibility of an alternative approach for improving the economics of the ABE fermentation by a co-production strategy. Actually, alternative ways for improving biobutanol production have attracted much attention during the last few years. *Escherichia coli* and *Saccharomyces cerevisiae*, two demonstrated genetic engineering platforms, have been exploited as hosts for the production of butanol in order to overcome the disadvantage of solventogenic clostridia [2, 21, 44]. However, the highest yield of *n*-butanol production in these systems is still lower than that in clostridia. It is well known that the overall economics of fermentation could be appreciately increased with the formation of a second product which could be easily recovered and which would be of higher value than the major products. Riboflavin was used as an example since it had been demonstrated that clostridial ABE fermentations could yield some riboflavin based on work published in the 1940s [35]. However, the riboflavin concentration in the clostridia culture was very low in these earlier systems. As shown in Fig. 1, *C. acetobutylicum* can use the carbon source for both solvent and riboflavin formation. If riboflavin could accumulate 0.5–1 g/l in ABE fermentation on an industrial scale, the process economics would be significantly improved. Here, we improved the *C. acetobutylicum* ATCC 824 strain by over-expression of its riboflavin operon *ribGBAH* and successfully produced 27 mg/l riboflavin in *C. acetobutylicum* culture broth in static flask. Preliminary optimized culture conditions improved the riboflavin production to more than 70 mg/l in flask. Thereafter, attempts were made to modify the *C. acetobutylicum* purine pathway by over-expression of the *Clostridium purF* gene, which encodes the rate-limiting enzyme PRPP amidotransferase, as defined in *Bacillus* [47], and its rational feedback resistant mutation version (*purFC*), for the improvement of riboflavin production.

Materials and methods

Chemicals and reagents

Riboflavin was purchased from Sigma–Aldrich (St. Louis, MO). All medium components were obtained from Difco (Detroit, MI) or Sigma–Aldrich. All restriction enzymes were obtained from New England Biolabs (NEB, Beverly, MA). The pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) was used for PCR product cloning. Automated DNA

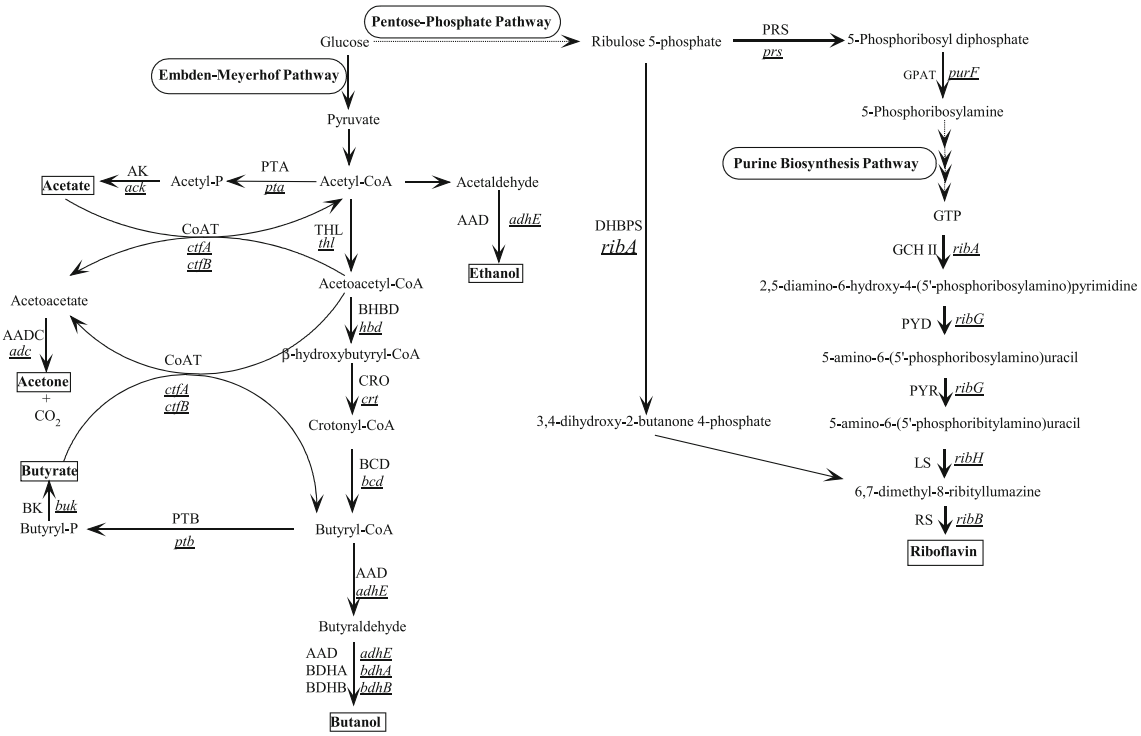

Fig. 1 Diagram of the *Clostridium acetobutylicum* metabolic pathway towards solvent and riboflavin production**.** Enzymes are listed as *uppercase letter* abbreviations; corresponding genes are in *italics* and *underlined*. Final fermentation products are *boxed*. *AK* Acetate kinase, *PTA* phosphotransacetylase, *THL* thiolase, *CoAT* CoA transferase, *AAD* alcohol/aldehyde dehydrogenase, *AADC* acetoacetate decarboxylase, *BHBD* hydroxylbutyryl dehydrogenase, *CRO* crotonase, *BCD* butyryl-CoA dehydrogenase, *BK* butyrate kinase, *PTB* phosphotransbutyrylase, *BDHA* butanol dehydrogenase isozymes A, *BDHB* butanol dehydrogenase isozymes B, *DHBPS* 3,4-DHBP synthase, *PRS* phosphoribosylpyrophosphate synthetase, *GPAT* glutamine phosphoribosylpyrophosphate amidotransferase, *GCH II* GTP cyclohydrolase II, *PYD* pyrimidine deaminase, *PYR* pyrimidine reductase, *LS* lumazine synthase, *RS* riboflavin synthase

sequencing was performed by LoneStar automated DNA sequencing (LoneStar Laboratories, Houston, TX).

Bacterial strains and plasmids

All bacteria strains are listed in Table 1 and plasmids are listed in Table 2.

Bacterial growth conditions

E. coli was grown aerobically at 37°C in Luria–Bertani (LB) medium, and *C. acetobutylicum* was grown anaerobically at 37°C in *Clostridium* growth medium (CGM) in a Forma Scientific anaerobic chamber (Thermo Forma, Marietta, OH). For *E. coli* recombinant strains, the medium was supplemented with ampicillin (100 μg/ml), chloramphenicol (35 μg/ml), kanamycin (50 μg/ml), and erythromycin (200 μg/ml) as appropriate. For the *C. acetobutylicum* strains, erythromycin (40 μg/ml for solidified agar plates and 100 μg/ml for liquid medium) and thiamphenicol (25 μg/ml) were used when necessary. For long-term storage, *E. coli* strains were cultivated and stored as glycerol stocks at −80°C. *C. acetobutylicum* strains were cultivated and stored as spore suspensions at 4°C or glycerol stocks at −80°C.

C. acetobutylicum fermentation

Single *C. acetobutylicum* colonies were inoculated into CGM medium with appropriate antibiotics and incubated overnight as seed cultures. An appropriate amount from the seed cultures was used for the subculture to yield a starting A_{600} of 0.1 for each fermentation experiment. During the fermentation process, the flasks were shaken periodically to improve cell growth. All cultures were carried out in triplicate except for those mentioned specifically.

Plasmid constructions and transformation

Plasmids from *E. coli* were purified using QIAprep Miniprep protocols (Qiagen, Valencia, CA), and those from *C. acetobutlycum* were purified according to the modified protocol [39]. Genomic DNA was purified from *C. acetobutylicum* using the Genomic DNA Purification kit from Puregene (Gentra Systems, Minneapolis, MN). All commercial enzymes used in this study (*Taq* polymerase,

Table 1 Bacterial strains

Strain	Description	Reference/source
Clostridium acetobutylicum		
824	Wild type	American Type Culture Collection (ATCC), Manassas VA
M5	pSOL1$^-$	[8]
Escherichia coli		
DH5α	F$^-$ *endA*1 *glnV*44 *thi*-1 *recA*1 *relA*1 *gyrA*96 *deoR nupG* Φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*) U169, *hsdR*17(rK$^-$ mK$^+$), λ^-	New England Biolabs (NEB), Beverly, Mass.
DH10*β*	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1endA1ara*D*139*Δ(*ara*,*leu*) *7697 galU galK* λ^- *rpsL nupG*	Gibco-BRL, Carlsbad, CA
BSV11	F$^-$, *glnV*44(AS) *LAM*$^-$ *mcrA*0 *rfbC*1 *endA1 ribB11::Tn5 spoT1 thi-1 mcrB9999 hsdR29*	Coli Genetic Stock Center (CGSC), New Haven, CT
BSV13	F$^-$, *glnV44*(AS) *LAM*$^-$ *mcrA0 ribA13::Tn5 rfbC1 endA1 spoT1 thi-1 mcrB9999 hsdR29*	CGSC, New Haven, CT
AB1117	Hfr(PO12) Δ(*gpt-proA)62 lacY1 or lacZ4 glnV44*(AS) *galK2*(Oc) *LAM*$^-$, *purF1 thi-1*	CGSC, New Haven, CT

Table 2 Plasmids used

Plasmid	Description[a]	Reference/ source
pAN1	CmR,Φ3TI methyltransferase, p15A ori	[31]
pDHKM	KmR, Φ3TI methyltransferase in pDHK29	[48]
pPTB	ErmR, shuttle vector	[48]
pJIR750	ThlR, shuttle vector, ColE1 ori, pIP404 ori II	[4]
pJpGN	P_{ptb}-ribGBAH-original was inserted into pJIR750	This study
pJpGC	P_{ptb}-ribGBAH-modified was inserted into pJIR750	This study
pJpGNA	P_{abrB}-ribA was inserted into pJpGN	This study
pJaA	P_{abrB}-ribA was inserted into pJIR750	This study
pJaF	P_{abrB}-purF was inserted into pJIR750	This study
pJaFC	P_{abrB}-purF-modified was inserted into pJIR750	This study
pJpGNF	P_{abrB}-purF was inserted into pJpGN	This study
pJpGNFC	P_{abrB}-purF-modified was inserted into pJpGN	This study

[a] P_{ptb}, *ptb* promoter; P_{abrB}, AbrB310 promoter; ribGBAH-original, original *rib* operon in *Clostridium acetobutylicum*; ribGBAH-modified, *ribG* and *ribB* start codons were changed to ATG in the *C. acetobutylicum rib* operon; ori, replicon origin for *Escherichia coli*; ori II, replicon origin for *Clostridium*; *Ap* ampicillin; *Em* erythromycin; *Thl* thiamphenicol; *Km* kanamycin; *Cm* chloramphenicol

restriction endonucleases, calf intestinal phosphatase, T4 DNA ligase, Klenow fragment of DNA polymerase I) were used according to the manufacturers' recommendations.

E. coli transformation followed the Sambrook protocol [37]. As to *C. acetobutylicum* transformation, plasmid DNA was methylated by the Φ3TI methyltransferase to prevent restriction by the clostridial endonuclease *Cac*824I [31]. This was achieved by transformation of the required plasmid into DH10*β E. coli* harboring vector pDHKM [48], carrying an active copy of the Φ3TI methyltransferase gene. Electrotransformation of *C. acetobutylicum* was carried out according to a modification of the protocol developed by Mermelstein [32], and positive transformants were isolated on agar-solidified CGM supplemented with the appropriate antibiotic. Transformations were also confirmed by plasmid DNA purification.

Oligonucleotide sequences are listed in Table 3. To construct the *rib* operon expression plasmids, we used the primer set ribG-For and ribG-Rev to amplify the original *rib* operon *ribGBAH* fragment (*ribG*: CAC0590; *ribB*: CAC0591; *ribA*: CAC0592; *ribH*: CAC0593) from *C. acetobutylicum* genomic DNA using *pfx* polymerase (Invitrogen). The 3.66-kb fragment was further cloned into the pPTB plasmid (pPpGN) and pJIR750 plasmid (pJpGN) after DNA sequencing confirmed the expected sequence in the pCR2.1-TOPO vector. As the *ribG* and *ribB* genes both use TTG as the start codon, these residues were changed to ATG using the QuikChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), and the mutations were confirmed by DNA sequence analysis. The pJpGC (harboring the mutated start codons in *ribGBAH*) plasmid was then constructed. *ribA* overexpression constructs were also made in the pJIR750 plasmid.

For the *purF* overexpression plasmids, the *purF* gene from *C. acetobutylicum* was placed under the control of the P_{abrB} promoter in the construction of plasmid pJaF. The QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) was used for introducing the two site replacements in the *purF* gene to give plasmid pJaFC. Similarly,

Table 3 Oligonucletides used in this study

Primers	Sequence[a]
ribG-For	5′-GGAGGATCCAGGAGGACCTTTCT*TTG*GAGATTTTTTTATTTG-3′
ribG-Rev	5′-GGCACTAGTCTATTCGCCAATTGTTTTAATTAAATTAG-3′
$P_{abrB310}$-For	5′-GGGTCTAGAAACAATCACCTCTTAAAACAATTATAC-3′
$P_{abrB310}$-Rev	5′-GTCGCGGCCGC CCTCCTTAAATTACATTATCCGACAAACA-3′
ribA-For	5′-GACGCGGCCGC*ATG*AATTTCAAATTTAATACTGTAG-3′
ribA-Rev	5′-GCTGGTACCCTCTCCTTTAAATTTCAATTAAGTG-3′
Rib-M1	5′-CGAGGATCCAGGAGGACCTTTCT*ATG*GAGATTTTTT-3′
Rib-M2	5′-ATACCTAAATAGGGGATGATTTGTT*ATG*TTTACCGGTATTATAGAG-3′
purF-For	5′-GCGGCCGCAAGAGAAGGAGATAGGT*ATG*-3′
purF-Rev	5′-GAGCTCTTAAGTTTCAAGTCTATCC-3′
purF-M1	5′-AGTAATTGGAGTACCTGTTTCAGGAATACCAGCGG-3′
purF-M2	5′-ACCGTATACTTTGGGGCTGATACAAAATAAATACATAGGTAGAACC-3′

[a] Restriction site sequence is underlined. The corresponding start codon in the primer sequence is given in italics

plasmids pJpGNF and pJpGNFC were constructed based on pJpGN.

Analysis of fermentation products

Cell growth was monitored by A_{600} with Beckman DU-800 spectrophotometer (Beckman Coulter, Fullerton, CA). Riboflavin was measured by A_{444} with a Beckman DU-800 spectrophotometer based on the modified protocol from Sauer [38]. Briefly, the fermentation broth was diluted with 0.1 M phosphate buffer and heated at 80°C for 10 min before centrifugation. The supernatant was then assayed. Similarly, culture broths were centrifuged immediately after sampling at 10,000*g* for 10 min at 4°C and the supernatant fluids then stored at −20°C for analysis. The concentrations of butanol, acetone, ethanol, butyrate, and acetate were determined by gas chromatography with a Hewlett-Packard 5890 Series II instrument (Hewlett-Packard, Palo Alto, CA) as described previously [48]. Glucose was measured by high-performance liquid chromatography (HPLC) as described [29].

Results

Construction of the riboflavin expression plasmids in *E. coli*

The riboflavin operon organization in *C. acetobutylicum* is the same as that in *B. subtilis* (Fig. 2), in which four genes, namely, *ribG*, *ribB*, *ribA*, and *ribH*, encoding five enzyme activities for riboflavin synthesis are clustered. The expression of the riboflavin operon, like that in many Gram-positive bacteria, is regulated by a conserved RNA regulatory element, RFN [16, 46]. Primers ribG-For and ribG-Rev were designed for PCR amplification of the *Ca-ribGBAH* fragment by excluding its own promoter and the RFN sequence. Also, the clostridial ribosome binding site AGGAGG was manually added upstream of the *ribG* gene on primer ribG-For. Taking into consideration that both *ribG* and *ribB* genes use the start codon TTG instead of ATG, mutation primers rib-M1 and rib-M2 were also used to prepare the modified fragment ribGBAH-C, which changed both start codons to ATG. After sequencing confirmed the accuracy of the PCR products, they were further cloned into the *E. coli–Clostridium* shuttle vector, pJIR750, for later expression analysis. Similarly, *ribA*, which has been reported to be very important in riboflavin expression [19], was cloned separately in the construct pJA, in which the P_{abrB} promoter directed *ribA* expression in pJIR750. An extra copy of the *ribA* expression cassette was placed downstream of the *ribGBAH* operon within pJpGN to yield pJpGNA.

Function of the riboflavin expression plasmids in *E. coli*

Expression of the *rib* operon or *ribA* gene in *E. coli* was verified by its complementation of *E. coli rib* mutants on M9 medium (Table 4). All *rib* operon overexpression constructs complemented both *E. coli ribA* and *ribB* mutants. However, the *ribA* overexpression construct pJA only complemented the *E. coli ribB* mutant.

The function of the riboflavin expression constructs was tested in *E. coli* cultures. *E. coli* DH5α with different constructs was shaken overnight at 200 rpm, 37°C in LB medium plus chloramphenicol. These cultures were inoculated at a ratio of 1 to 100 into 250-ml flasks containing 50 ml of LB plus chloramphenicol and shaken at 200 rpm, 37°C for 48 h before measuring riboflavin production. As shown in Fig. 3, cell growth was not significantly different between strains bearing different constructs. Overexpression of the *rib* operon released riboflavin into the culture medium, which was observable as a yellowish color in the supernatant. No significant difference was observed

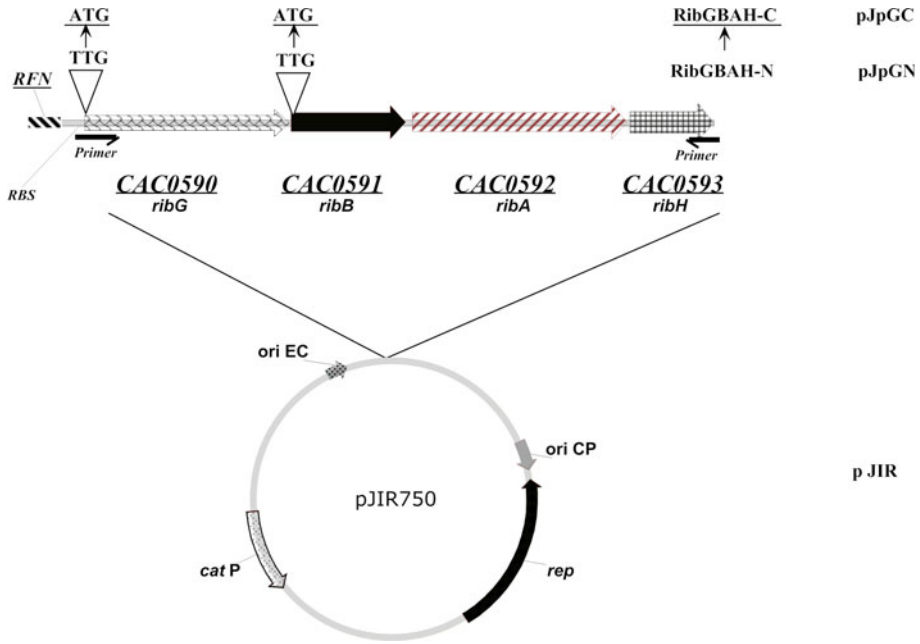

Fig. 2 Illustration of the *Escherichia coli–Clostridium* plasmids for riboflavin overexpression. Riboflavin operon *ribG* (CAC0590), *ribB* (CAC0591), *ribA* (CAC0592), and *ribH* (CAC0593) organization is shown at *top* of figure. The PCR primer binding position for *ribGBAH* fragment amplification that excluded the promoter, RBS, and RFN sequence is shown. The start codon difference between pJpGC and pJpGN is also illustrated. *Ori EC E. coli* replication origin, *Ori CP Clostridium* replication origin, *cat P* chloramphenicol resistance gene from *C. perfringens*, *rep* replication gene from pIP404

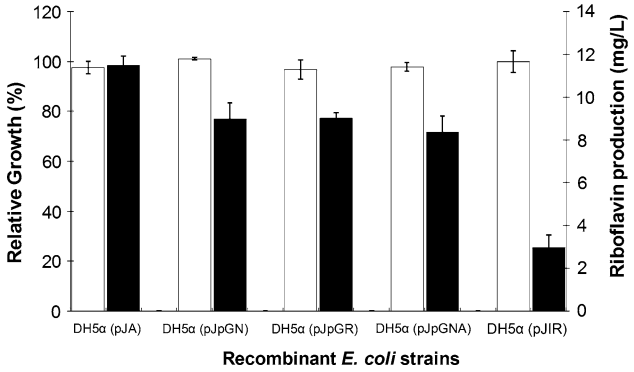

Fig. 3 Comparison of riboflavin production and growth in recombinant *E. coli* strains. Overnight recombinant *E. coli* strains were subcultured into 50-ml Luria–Bertani (LB) medium plus antibiotic in 250-ml flasks and shaken for 48 h at 200 rpm, 37°C. Growth was measured at A_{600}, and riboflavin in the supernatant was measured at A_{444}. Growth is shown as the percentage of the parent DH5α(pJIR) culture (*open columns*), and riboflavin is shown as milligrams per liter in culture broth (*filled columns*)

between DH5α/pJpGN and DH5α/pJpGC in terms of amount of riboflavin released, although a slightly lower riboflavin production was observed in DH5α/pJpGNA, which contained an extra copy of *ribA*. Interestingly, DH5α/pJA yielded the best riboflavin production under these culture conditions.

Function of the riboflavin expression plasmids in *C. acetobutylicum* ATCC 824

C. acetobutylicum produces riboflavin under the appropriate conditions. In recent years, the production of riboflavin in milk media has been used as a trait for characterizing *C. acetobutylicum* [23]. However, under standard laboratory culture conditions in CGM medium, *C. acetobutylicum* ATCC824 does not produce riboflavin to a detectable amount in the medium. After methylation by the plasmid pDHKM in *E. coli* DH10*β*, the riboflavin expression plasmids together with control plasmid pJIR were transformed into *C. acetobutylicum* ATCC 824 by electroporation. After a 2-day incubation, all transformation plates showed colonies, and *C. acetobutylicum* ATCC 824(pJpGN), *C. acetobutylicum* ATCC 824(pJpGC), and *C. acetobutylicum* ATCC 824(pJpGNA) all secreted a yellowish color, apparently riboflavin on the plate. However, transformation of *C. acetobutylicum* ATCC 824 with plasmid pJA, which showed the best riboflavin production in *E. coli*, did not produce any riboflavin on the plate, unlike the other riboflavin constructs. The *C. acetobutylicum* ATCC 824(pJA) colony remained white in color even after a 1-week incubation and appeared the same as the vector-containing strain, *C. acetobutylicum* ATCC 824(pJIR).

Randomly picked colonies from the freshly grown plates described above were inoculated into CGM medium with thiamphenicol and incubated overnight as seed cultures, which were then subcultured into 40 ml CGM plus thiamphenicol in a 50-ml plastic tube (flask). The starting A_{600} of the cultures was set as 0.1 to give each culture a comparable growth profile. After 3 days of static culture (the tubes were mixed by inversion a few times each day during the culture process), both riboflavin production and solvent production were assayed.

As seen from Fig. 4, *C. acetobutylicum* ATCC 824(pJpGN), *C. acetobutylicum* ATCC 824(pJpGC), and *C. acetobutylicum* ATCC 824(pJpGNA) all produced a

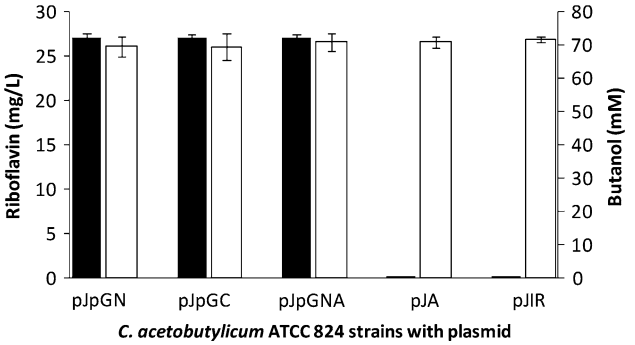

Fig. 4 Comparison of riboflavin production and butanol production in recombinant *C. acetobutylicum* ATCC 824 strains. Recombinant strains cultured overnight were subcultured into 40 ml *Clostridium* growth medium (CGM) plus antibiotic in 50-ml tubes and incubated statically at 37°C under anaerobic conditions. *C. acetobutylicum* ATCC 824(pJIR) and *C. acetobutylicum* ATCC 824(pJA) were tested in triplicate, while all other recombinants were examined in at least four repeats. The average and standard derivations for riboflavin production (*filled columns*) and butanol production (*open columns*) are shown

Table 4 Complementation of *E. coli rib* mutants by riboflavin over-expression constructs

Recombinant plasmids	*E. coli* hosts	
	BSV 11 (*ribB*$^-$)	BSV 13 (*ribA*$^-$)
pJIR	–	–
pJA	+	–
pJpGN	+	+
pJpGC	+	+
pJpGNA	+	+

significant amount of riboflavin in the medium, which took on a bright yellow color. However, there is no significant difference between these three constructs in terms of riboflavin production. Taking this result into consideration as well as the finding that overexpression of *ribA* alone in *C. acetobutylicum* ATCC 824 did not produce riboflavin at all, we focused mainly on *C. acetobutylicum* ATCC 824(pJpGN) in subsequent experiments.

It is well known that culture conditions affect product formation during fermentation. An excess amount of ferrous ions in the medium is known to inhibit riboflavin accumulation in some of the natural producers, which include *C. acetobutylicum* [35]. To verify whether 15 mg/l ferrous sulfate in CGM medium can be considered to be "in excess" in terms of affecting riboflavin production, we tested the effects of different amounts of ferrous sulfate (range 3–15 mg/l) in CGM medium on growth, riboflavin production and solvent production (Fig. 5).

When only a low amount of iron was added to the medium, a lower cell growth was obtained and a lower concentration of butanol accumulated in the broth. This result indicates that 15 mg/l ferrous sulfate is necessary for optimal cell growth. Omission of all five trace salts (ferrous sulfate, calcium chloride, magnesium sulfate, zinc chloride, cobalt chloride) in the CGM medium resulted in very weak cell growth and the loss of solvent production. As shown in Fig. 5, there was no significant difference in riboflavin production when ferrous sulfate concentrations ranged from 3 to 15 mg/l. The addition of 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer to the CGM medium by setting the pH to 5.5 greatly improved both riboflavin and solvent production by strain *C. acetobutylicum* ATCC 824(pJpGN), as shown in Fig. 6: riboflavin increased from 27 to 70 mg/l, while butanol increased from 70 to 190 mM, which is comparable to the level found in bioreactors [26]. Under the static culture condition with CGM medium, the pH of the *C. acetobutylicum* culture medium could drop below 4.0 during the active growth phase. The addition of MES buffer controlled the culture pH during the culture process, and residual glucose in the medium was reduced greatly. It is worth noting that the co-production of riboflavin exerted no effect on solvent production (Table 5).

Over-expression of *purF* did not improve riboflavin production

The direct precursor for riboflavin bio-synthesis is GTP, which originates in the purine pathway. It is well recognized that glutamine-phosphoribosyl-pyrophosphate (PRPP) amidotransferase plays a significant role in purine pathway regulation [42]. To further improve riboflavin expression in *C. acetobutylicum*, *Ca-purF*, which encodes glutamine-PRPP amidotransferase, was manipulated to test its effect on riboflavin production. Early attempts to increase the biosynthesis of purine nucleotides focused on the deregulation of genes encoding pathway enzymes. It has recently been recognized that the enzymatic activity of glutamine-PRPP amidotransferase is inhibited by the pathway end-products, and the desensitization of *purF* has been achieved in several species [22, 41, 47]. Therefore, overexpression of both the original *purF* and desensitized *purF* were examined for their effect on riboflavin production in *C. acetobutylicum*.

Similarly, pJIR750 was chosen as the backbone plasmid for *purF* overexpression. Using *C. acetobutylicum* ATCC 824 genomic DNA as a template, the *purF* gene was amplified and cloned into pCR2.1-TOPO vector for sequencing. The QuikChange Multi Site-Directed Mutagenesis kit was used to introduce mutations in *purF*, which resulted in the mutated version *purFC* with two amino acid replacements, D302V and K325Q. As shown in Fig. 7, the alterations correspond to those found in feedback resistant *purF*

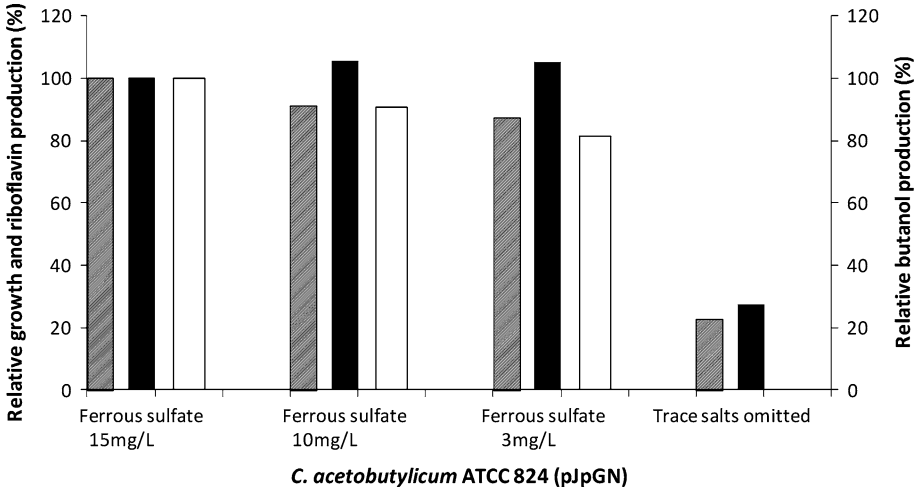

Fig. 5 Relatively high iron was found to be necessary for *C. acetobutylicum* ATCC 824 riboflavin and solvent production. *C. acetobutylicum* ATCC 824(pJpGN) was grown overnight in CGM medium as a seed culture, centrifuged, washed with phosphate buffer under anaerobic conditions, and then subcultured into different media. The cultures were statically incubated at 37°C for 3 days before the riboflavin and solvent concentrations were measured. Relative growth (*pattern filled columns*), relative riboflavin production (*filled columns*), and relative butanol production (*open columns*) are all shown relative to the value of the highest ferrous sulfate culture

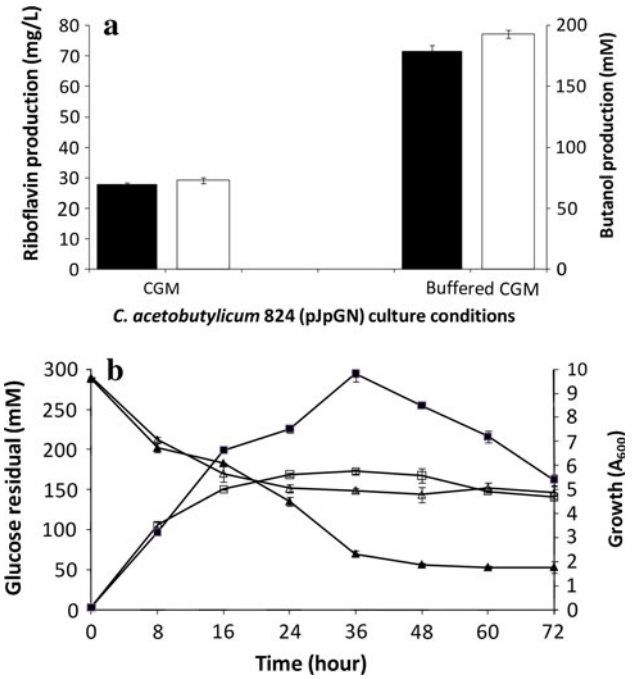

Fig. 6 Maintenance of culture pH improved both riboflavin and solvent production in *Clostridium* cultures. *Clostridium* cultures were tested in either regular CGM medium or buffered CGM medium which contained 50 mM MES at pH 5.5. Flasks were incubated statically for 3 days prior to measuring riboflavin and solvent production. **a** *Filled column* Riboflavin production, *open column* butanol production. **b** *Square points* Growth, *triangles* glucose concentration. *Open symbols* Regular CGM medium, *filled symbols* buffered CGM medium

variants in other organisms since the amino acid sequence of PurF remains conserved among many species [22, 41]. A strong clostridial promoter, P_{abrB}, was used to direct *purF* and *purFC* expression in the constructed plasmids, pJaF and pJaFC. Both plasmids complemented an *E. coli purF* mutant. However, neither plasmid resulted in an improvement in riboflavin production. The *purF*/*purFC* expression cassette was also introduced into pJpGN to yield pJpGNF and pJpGNFC, which co-expressed the *rib* operon and *purF*/*purFC* simultaneously. However, when introduced into *E. coli*, no improvement was observed for either construct. Instead, both constructs resulted in slightly reduced riboflavin production compared to the control strain with only pJpGN overexpressed (Fig. 8a).

Methylated plasmid pJpGNF and pJpGNFC were introduced into *C. acetobutylicum* ATCC 824 by electroporation, and apparent secretion of riboflavin on plates was observed for both kinds of recombinants. However, with respect to riboflavin production in buffered CGM medium, a slightly reduced riboflavin accumulation was observed for both strains bearing plasmids over-expressing either regular *purF* or desensitized *purFC* (Fig. 8b).

Discussion

Researchers have been attempting to improve the ABE fermentation process since its discovery, with the aim of making it more cost effective and efficient in industrial-scale applications. Different carbon sources have been tested for the possibility of increasing solvent production and reducing process costing, including corn, potato, rice, wheat, molasses [35], and milk industrial by-products, such as whey [28], cellulosic feedstock [12], and even civil waste [7]. Experiments using these different raw materials

Table 5 Production and yield of solvent in recombinant *Clostridium* cultures

Culture medium	Recombinant *Clostridium* strain	Production (mM)		Yield[a] (mM/mM)	
		Butanol	Total solvent	Butanol	Total solvent
CGM	824(pJIR)	71.46	101.82	0.49	0.70
	824(pJpGN)	72.84	101.7	0.50	0.70
CGM-buffered	824(pJIR)	190.68	332.85	0.72	1.26
	824(pJpGN)	193.22	330.69	0.72	1.23

CGM Clostridium growth medium

[a] Yield calculation based on the solvent produced and glucose consumed in the 3-day flask cultures, as shown in Fig. 6a

revealed that notable amounts of riboflavin were produced in the culture broth in some systems but not in others. In recent years, in addition to improving the solvent production ability, advanced fermentation techniques and downstream processing technology have been extensively studied in terms of ABE fermentation for the purpose of reducing process costing [11, 13]. It has also been recognized that ABE fermentation by-products, such as hydrogen, carbon dioxide, and distillation residue, contribute significantly to the process economics [36]. Therefore, the production of any number of high-value chemicals as by-products during ABE fermentation would definitely improve the economic perspective of the traditional ABE fermentation process. Riboflavin, which is widely used as an additive to animal feed and food products and as a medicine, has an annual market of several million kilograms [43]. Considering the economical analysis of the butanol fermentation [36] as well as the more favorable price of biofeedstock versus oil in recent years, the co-production of riboflavin in amounts of 0.5–1 g/l together with solvent production would double the value of the culture products on a per-liter basis.

We have demonstrated the possibility of improving ABE fermentation through the co-production of riboflavin and solvent. We over-expressed the *C. acetobutylicum* riboflavin operon by placing the *ribGBAH* gene cluster under the direction of the *Ca*-P_{PTB} promoter using the *E. coli–Clostridium* shuttle vector pJIR750. The riboflavin production was elevated in both *E. coli* and clostridia hosts by the plasmid pJpGN. Altering the start codon in *ribG* and *ribB* did not demonstrate a positive effect on riboflavin production. *Bacillus subtilis* is the most extensively studied Gram-positive bacterium that has been used as a reference in the study of clostridia molecular biology. The gene organization of the *Clostridium* genomic DNA resembles that of *B. subtilis* in many aspects. The same gene cluster was confirmed in *C. acetobutylicum*, showing a high similarity to *B. subtilis* riboflavin genes in amino acid sequence. The bi-functional *Bs-ribA* gene, which encodes both GTP cyclohydrolase II and 3,4-DHBP synthase, complements both *ribB*$^-$ and *ribA*$^-$mutants in *E. coli* [19]. In addition, *Bs-ribA* encodes the rate-limiting enzymes in an industrial riboflavin-producing strain: an extra copy of *ribA* introduced into the genome was found to increase the riboflavin titer by 25% [19]. However, in our experiments, the over-expression of an extra copy of *ribA* (pJpGNA) did not improve riboflavin production in either *E. coli* or *C. acetobutylicum*. Furthermore, single over-expression of *ribA* (pJA) produced very low levels of riboflavin in *C. acetobutylicum* cultures, although this construct was the best for riboflavin production in *E. coli* cultures. These results suggest that the *Ca-ribA* functions differently than that in *B. subtilis*. This conclusion is also supported by the fact that *Ca-ribA* did not complement the *E. coli ribA*$^-$ mutant. The *ribA* (denoted *ribBA*) in Gram-negative *Helicobacter pylori* strain P1 has been reported not to complement a *E. coli ribA*$^-$ mutant. Homology between *Hp*-ribA and *Ec*-ribA was found to be very low, and an extra copy of *ribA* encoding a functional GCH II enzyme was found in the *H. pylori* genome [14]. However, in *C. acetobutylicum* ATCC 824, the amino acids 207–399 in the RibA protein, the fragment which comprises the GCH II function, showed a high homology to *Ec*-RibA (52% identical); this homology is even higher than that of DHBP synthase (41.3% identical). Furthermore, no extra *ribA* gene was found in the *C. acetobutylicum* genome and mega plasmid by BLAST search. The reason for this phenomenon observed in *Ca-ribA* function remains unknown, and it may deserve detailed study.

Culture conditions significantly affect riboflavin production. The recombinant 824(pJpGN) greatly increased its riboflavin production, even in the static flask culture, following modification of the composition of the medium. It has been known since the 1930s that culture conditions affect riboflavin production in *C. acetobutylicum* [9, 35], with different raw materials resulting in significant differences in riboflavin production, ranging from trace amounts to 7 mg/g dried residue [5, 35]. Iron was very early characterized as one of the major effectors of riboflavin accumulation in *C. acetobutylicum*, and this was later explained as iron inhibiting riboflavin production by destroying riboflavin through peroxide mechanisms [27]. The iron concentration range of 1–2 mg/l was supposed to be the optimal level

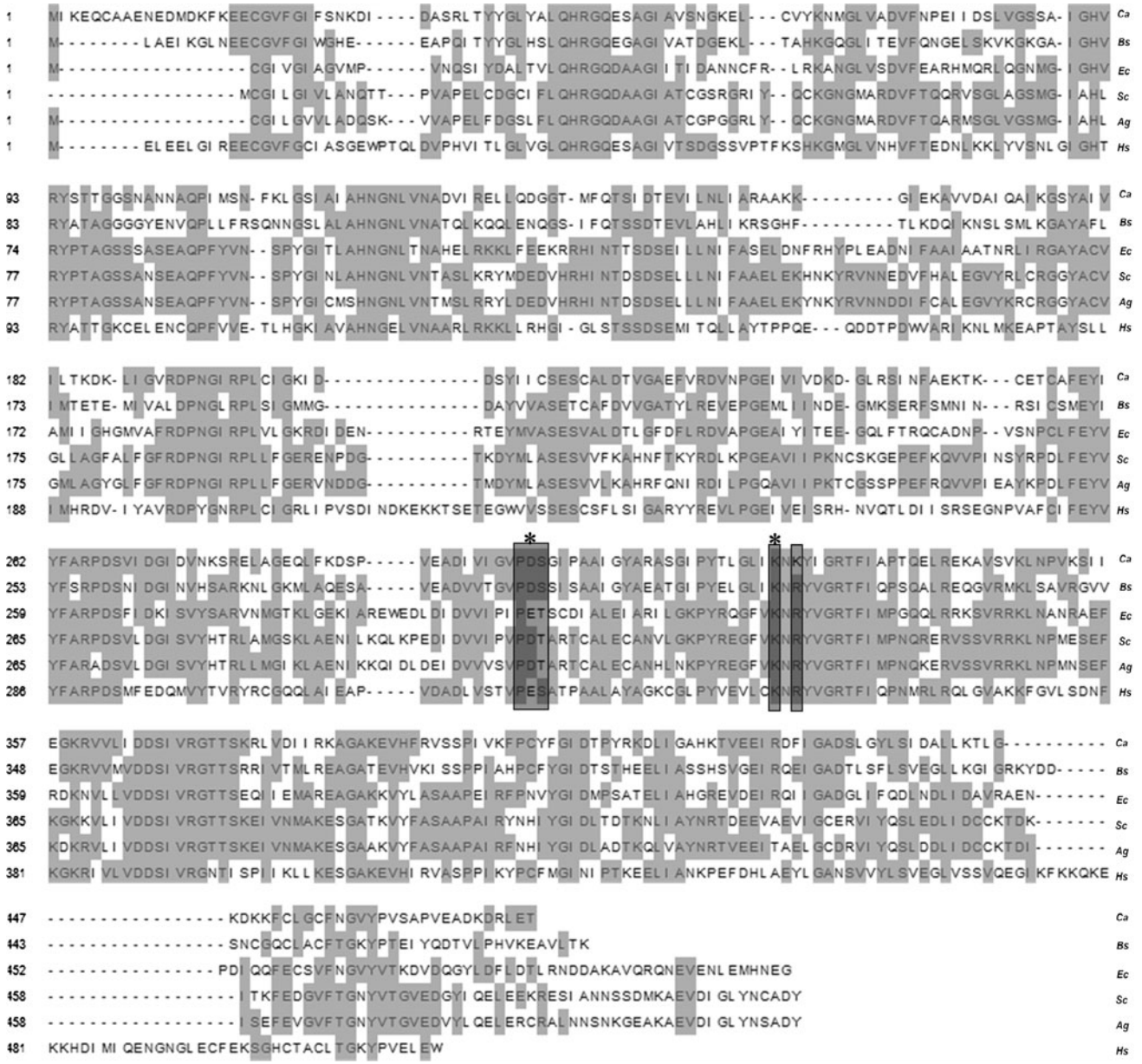

Fig. 7 Alignment comparison between different species revealed highly conserved phosphoribosyl-pyrophosphate (PRPP) amidotransferase. The amino acid sequences were aligned using the Clustal-W program. Identical residues are *shaded*, while conserved sequences targeted for modification in the literature are *boxed*. Residues replaced in this study to desensitize PRPP amidotransferase are marked with *asterisk*. *Bs Bacillus subtilis*, *Ca Clostridium acetobutylicum*, *Ec Escherichia coli*, *Sc Saccharomyces cerevisiae, Ag Ashbya gossyppii*, *Hs Homo sapiens*

for riboflavin accumulation [18]. *C. acetobutylicum* ATCC 824 does not accumulate any detectable amount of riboflavin in CGM medium even though more than 10 mg/l riboflavin was observed in milk and corn mash medium after prolonged incubation. The addition of 15 mg/l ferrous sulfate heptahydrate to CGM medium was expected to result in an inhibition of riboflavin production since this concentration is much higher than the reported optimum amount. However, decreasing the iron amount in the standard CGM media did not significantly improve riboflavin production for 824(pJpGN), and the small increase in riboflavin production came at the expense of growth and solvent production (Fig. 5). If trace salts were omitted, the cell growth was severely limited and very little solvent and riboflavin was produced. It is well known that strains perform better in controlled bioreactors than in static flask cultures. We found that the culture pH dropped to <4.0 during the active growing phase in static flasks with CGM medium, which may limit culture productivity. The addition of Good′s buffer, MES, resulted in a striking improvement in both

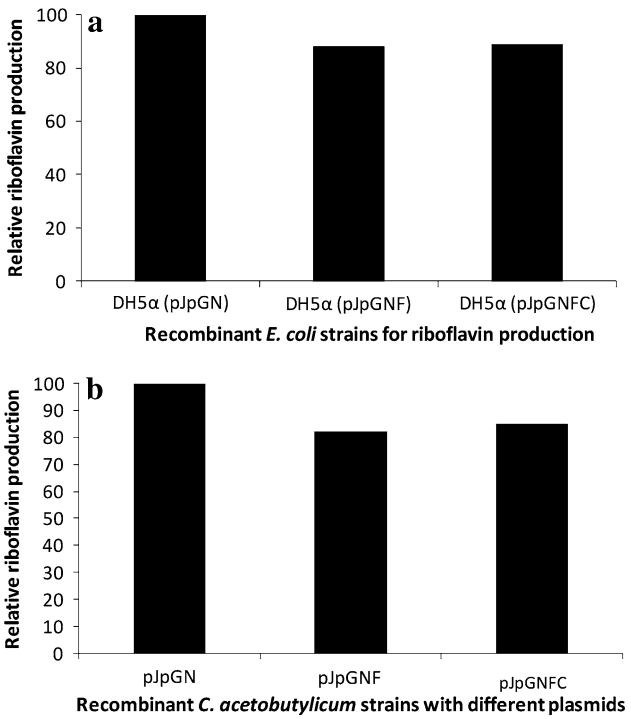

Fig. 8 Co-expression of *purF* and desensitized *purFC* with the *rib* operon did not improve riboflavin production. *E. coli* cultures were shaken in LB medium plus kanamycin at 37°C for 2 days before riboflavin measurement (**a**), and *Clostridium* cultures were tested in buffered CGM supplemented with thiamphenicol for 3 days (**b**)

riboflavin and solvent production: riboflavin production was improved by 160% and butanol production increased by 170%; these values are comparable to those found in bioreactor studies [26]. The significant improvement could be explained by the pH buffering capacity and the observed better growth and more complete glucose consumption in the buffered medium (Fig. 6).

The addition of GTP, the precursor of riboflavin synthesis, to the medium improved riboflavin production in different organisms, indicating the possibility of metabolic engineering of the purine pathway with the aim of increasing the production of purine products and riboflavin. However, purine biosynthesis is regulated simultaneously at the levels of gene transcription, translation, and enzymatic activity. Among the steps in the complicated purine biosynthesis pathway, PRPP amidotransferase has been demonstrated to be the rate-limiting step. It has remained conserved in different organisms and has therefore been the target of metabolic engineering for improving riboflavin production. PRPP amidotransferase activity is subject to synergistic feedback inhibition through the specific binding of adenine and guanine nucleotides to specific amino acids in the enzyme [33, 42, 49]. In *E. coli*, amino acid substitutions of K326Q or P410W have been shown to release PRPP amidotransferase from feedback inhibition [49]. Shimaoka et al. [41] demonstrated that the singly mutated K326Q PurF enzyme was desensitized in feedback inhibition and that the strain's inosine production ability was significantly enhanced. In *B. subtilis*, a similar de-regulation phenomenon was observed by introducing amino acid mutations S283A or K305Q or R307Q or S347A [47]. Most recently, a similar strategy was used for *Ashbya gossyppii* in improving riboflavin production. The introduction of the double mutations K333Q and D310V in *Ag-ade*4 resulted in an increase of riboflavin production from 80 mg/l to more than 200 mg/l in shake flask cultures [22]. It is noteworthy that the synergistic function sites within PurF are conserved in many organisms, appearing in the *Ca*-PurF sequence which is highly conserved with *Bs*-PurF (Fig. 7). In *B. subtilis* PurF, disruption of the P281-*cis*-D282-S283 structure has been found to significantly deregulate the synergistic feedback inhibition [47]. The *B. subtilis* equivalent K305Q mutation has been shown to consistently improve the production of products of the purine pathway. In our pJaFC construct, both the D302V and K325Q mutations were adopted to mimic the equivalent mutations of D^{282} and K^{305} in *B. subtilis*. However, neither over-expression of the original *purF* nor the desensitized *purFC* improved riboflavin production in our experiments. One possible explanation is that strong overexpression of the limiting enzyme in the purine biosynthesis imposed an additional metabolic burden on the host cells which may, in turn, have induced stress response reactions and other undesirable negative effects on the cells' physiology. The P_{abrB} promoter is a strong *Clostridium* promoter that has been found to function better than the P_{PTB} promoter in *C. acetobutylicum* in earlier studies [40]. However, replacing the P_{abrB} promoter by the P_{PTB} promoter for *purFC* expression plasmid did not improve its performance (data not shown). Efforts to assay *Clostridium* glutamine-PRPP amidotransferase activity were not successful, even with partially purified cell lysate after ammonium sulfate precipitation: only very weak activities was detected in recombinant *E. coli* 1117 (pJaF) and 1117 (pJaFC), although 1117 (pJaFC) showed higher activity than 1117 (pJaF). The low activity could be explained by the instability of amidotransferase, as demonstrated in *B. subtilis* [45]. Compared to *B. subtilis*, *Clostridium* amidotransferase may be more sensitive to oxygen. However, it was confirmed that both *purF*/*purFC* function well in vivo in the complementation of *E. coli purF* mutants. It could be seen that riboflavin produced in *C. acetobutylicum* remains much lower than that in engineered *B. subtilis*. This could be due to the process used and could also be explained by the fact that the *B. subtilis* host used for riboflavin production was developed by the more classical strain improvement approaches, which resulted in purine biosynthesis deregulation at the transcription and translation levels. Studies in *Lactococus lactis* confirmed that traditional mutagenesis strain screening can result in mutations in the *rib*

operon regulation area [6]. Another reason for the low riboflavin production in *C. acetobutylicum* might be due to the low cell density obtained in the static culture, with biomass in terms of A_{600} generally being less than 10. In comparison, in *B. subtilis*, fed-batch cultures in bioreactors can accumulate biomass up to 50 g/l [25]. Improvement of the culture conditions with the aim of increasing biomass could definitely be an approach to effect an increase in riboflavin concentration in *C. acetobutylicum* cultures. As shown in *Pichia pastoris*, over-expressing the *rib* operon led to 20 mg/l riboflavin in flasks. The same strain could produce more than 120 mg/l in a fed-batch bioreactor [30]. The major concern for a co-production process is whether the second product affects the yield of the major product. In our study, no effect on solvent production was observed when 100 mg/l riboflavin was present in the culture medium (data not shown); this concentration is about the saturation amount of riboflavin in the medium. Thus, increased levels of riboflavin in the medium should not exert extra burdens on cell metabolism. In our experiments, riboflavin accumulated up to 70 mg/l in static flask cultures without affecting solvent production in *C. acetobutylicum* ATCC 824(pJpGN), which supports the rationale of co-production of a high-value product in improving the economics of the ABE fermentation process. When better host strains and substrates are used, it can be expected that higher amounts of riboflavin will be produced during ABE fermentation, especially under controlled optimal bioreactor conditions.

Interestingly, it was found that the gene regulation mechanisms in *C. acetobutylicum* differ from those of its cousin *B. subtilis* in terms of riboflavin and purine pathway biosynthesis. The molecular biology study of *C. acetobutylicum* has been carried out by following the lead of *B. subtilis*. However, there may be subtle differences in various regulatory systems between these two Gram-positive organisms that need to be taken into consideration in studies of *C. acetobutylicum*.

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