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Novel expression system for *Corynebacterium acetoacidophilum* and *Escherichia coli* based on the T7 RNA polymerase-dependent promoter

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Abstract The industrially important species of corynebacteria viz. *Corynebacterium acetoacidophilum* appear to be alternative hosts for recombinant protein production; despite many efforts, a strong promoter-based system in corynebacteria has not been established so far. Described here is a T7 promoter-based expression system which was functional in both gram-positive *C. acetoacidophilum* and gram-negative *Escherichia coli* in an external inducer independent manner. This is the very first report of a T7 expression system for *Corynebacterium* sp. Also, it is a useful addition in the existing T7 expression systems of *E. coli*.

Keywords *Corynebacterium* · T7 RNA polymerase · T7 promoter · FtsZ promoter · Protein expression system · *E. coli*

Introduction

In the last two decades, the use of recombinant proteins has greatly increased both at industrial and laboratory levels. A large number of putative genes are continuously added to the GenBank, and to decipher the function of these unknown genes we need to have novel expression tools for high throughput screening of heterologous protein expression.

To date, many vector systems have been developed for a broad range of organisms, but bacterial systems remain the most attractive due to its low cost, high growth rate, and easy handling. The gram-negative bacterium *Escherichia coli* is the most commonly used organism for heterologous production of proteins due to the fact that its genetics is well studied and is an established system.

In *E. coli*, the T7 expression system (Studier and Moffatt 1986) is the most popular and extensively used system for high-level protein production. In this system, a plasmid contains an expression cassette in which a gene of interest is inserted downstream to an extremely strong promoter from the *E. coli* bacteriophage T7. This promoter is recognized only by its own RNA polymerase, the T7 RNA polymerase, encoded by *gene 1* of bacteriophage T7. The T7 RNA polymerase is highly processive which does not recognize promoters of the host; consequently, it leads to a high level expression of the target protein. For expression, the T7 plasmid carrying gene of interest under T7 promoter is transformed into a specific host, e.g., *E. coli* BL21 (DE3), that contains a single copy of T7 RNA polymerase gene under control of lacUV5 promoter as a lambda DE3 lysogen which is induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Despite the fact that protein expression is dependent on the inducer IPTG, this system has proven as a valuable tool in molecular biology, and the usefulness of this system in *E. coli* cannot be underestimated. But, IPTG is very expensive and toxic to the host which in many instances greatly reduce the growth rate of the host (Makrides 1996).

Although a high level of heterologous proteins are routinely produced through this system in *E. coli* both at industry and laboratory levels, one of the major disadvantages of this organism is that it is unable to efficiently secrete target protein in the medium; thus, it cannot be used for extracellular production of heterologous proteins. Moreover, this organism is not a suitable host to express proteins to be used for therapeutic and pharmaceutical purposes due to the accumulation of lipopolysaccharide (LPS), generally referred to as endotoxins,

Jahar Kanti Deb is deceased.

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which are pyrogenic in humans and other mammals. Recombinant proteins produced in *E. coli* must be purified in second step to become endotoxin-free prior to their therapeutic uses (Perola et al. 2007). In this context, GRAS members of the gram-positive coryneform bacteria are able to efficiently secrete proteins in the medium (Krämer 1994). These are facultative aerobic soil bacteria with high GC content. The members of this genus may also target folded proteins into the extracellular environment using their twin-arginine translocation (TAT) pathway (Kikuchi et al. 2006) and are thus best suited for being used for production of proteins of therapeutic uses.

Corynebacterium glutamicum and related species have been widely used for industrial production of amino acids. *C. glutamicum* itself produces more than 1.5 million tons of glutamate and 0.75 million tons of lysine each year (Kimura 2003). Despite immense industrial importance of *C. glutamicum* and related species, their usage as expression host for high-level protein production is still limited.

Few recombinant proteins have been expressed in *C. glutamicum* and related species which includes α-amylase of *Bacillus amiloliquefaciens* (Smith et al. 1986), cellulase of *Cellulomonas fimi* (Paradis et al. 1987), nuclease of *Staphylococcus aureus* (Liebl et al. 1992), lactose permease of *E. coli* (Brabetz et al. 1993), ovine interferon gamma as fusion protein with glutathione *S*-transferase (Billman-Jacob et al. 1994), protease (*bprV*) of *Dichelobacter nodosus* (Billman-Jacob et al. 1995), subtilisin (*aprE*) of *Bacillus subtilis* (Billman-Jacob et al. 1995), streptokinase as fusion with glutathione *S*-transferase (Srivastava and Deb 2002), transglutaminases of *Streptoverticillium mobaraense* (Kikuchi et al. 2003; Date et al. 2003) and *Streptomyces mobaraensis* (Date et al. 2004; Itaya and Kikuchi 2008), and human epidermal growth factor (Date et al. 2006). The promoters used so far for protein expression are native promoters of corynebacteria itself. Heterologous *E. coli* promoters (*lac*, *trc*, and *ara*) have been found to be functional in corynebacteria, but native as well as *E. coli* promoters are not strong enough in corynebacteria to serve the purpose for high-level protein expression (Pátek et al. 2003). The use of sugar-inducible promoters (*lac* and *ara*) of *E. coli* in *Corynebacterium* spp. are of limited use as the latter completely lack the lactose and arabinose metabolizing pathways (except *C. glutamicum* ATCC 31831 which grows on L-arabinose as sole carbon source; Kawaguchi et al. 2009), and thus they lack transporters of their corresponding inducers. Cloning of transporter for corresponding inducer did not give very positive results.

In the present study, we developed a two-plasmid expression system for corynebacteria based on T7 RNA polymerase-dependent promoter. Its function was shown in both gram-positive *C. acetoacidophilum* and gram-negative *E. coli*. It might be also used for high-level production of heterologous proteins in *C. glutamicum* and other related corynebacteria.

Materials and methods

Bacterial strains, media, and growth conditions

The bacterial strains used in this study are shown in Table 1. *E. coli* strains were grown aerobically on a rotary shaker (180–200 rpm) at 37 °C in Luria–Bertani (LB) broth or LB supplemented with 1.5 % agar on plates. The corynebacteria strain used in present study was *C. acetoacidophilum* ATCC 21476 (Deb et al. 1990) which is very closely related with *C. glutamicum* as evident from sequencing of 16S rRNA gene and species specific islands (SSIs) sequences. *C. acetoacidophilum* were grown at 30 °C in brain heart infusion (BHI) broth or BHI–agar. The antibiotics kanamycin and chloramphenicol wherever required were used at concentrations 50 μg ml^{-1} and 30 μg ml^{-1} for *E. coli* and 10 μg ml^{-1} and 6 μg ml^{-1} for *C. acetoacidophilum*, respectively.

Chemicals and enzymes

All chemicals for this study were purchased from either Sigma-Aldrich (St. Louis, USA) or Merck (Darmstadt, Germany). Restriction enzymes, DNA polymerases, ligase, and other DNA modifying enzymes were purchased from either New England Biolabs (MA, USA) or Fermentas (MA, USA).

Isolation and modification of plasmid and genomic DNA

All plasmids used in present study are listed in Table 2. Plasmid DNA from *E. coli* was isolated using QIAprep Spin Miniprep kit (Qiagen, Germany) following the manufacturer's instructions. Plasmid DNA from *C. acetoacidophilum* was isolated manually by the method described by Mukherjee et al. (1990). Genomic DNA from *C. acetoacidophilum* was isolated using QIAamp Tissue Kit (Qiagen). All DNA manipulations were carried out by standard procedures (Sambrook et al. 1989) and the instructions provided by the material suppliers.

Transformation and electroporation of plasmid DNA into host cell

Chemically competent *E. coli* cells were transformed with plasmid DNA by heat shock method (Cohen et al. 1972). *C. acetoacidophilum* cells were made electrocompetent, and subsequently electroporation of plasmid DNA were carried out by methods described by van der Rest et al. (1999).

Table 1 List of bacterial strains used in this study

Bacterial strain	Relevant characteristics	Reference
Escherichia coli		
Top10	*mcr*A, Δ(*mrr*⁻*hsd*RMS⁻*mcr*BC), Δ*lac*X74, *deo*R, *rec*A1, ar*a*D139Δ(*ara-leu*)7697, *gal*K, *rps*L, *end*A1, *nup*G	Invitrogen
BL21(DE3)	BL21 carrying the lambda DE3 lysogen	Novagen
Top10(*k*RNAP)	Top10 harboring plasmid pJE-*k*RNAP, Km^r	This study
Top10(*fts*RNAP)	Top10 harboring plasmid pJE-*fts*RNAP, Km^r	This study
Corynebacterium acetoacidophilum ATCC 21476		
B 30st	A streptomycin-resistant mutant of *C. acetoacidophilum* ATCC 21476	Deb et al. 1990; Karan 2000; Jana et al. 2005
B 30st(*k*RNAP)	B 30st harboring plasmid pJEC-*k*RNAP, Stp^r, Km^r	This study
B 30st(*fts*RNAP)	B 30st harboring plasmid pJEC-*fts*RNAP, Stp^r, Km^r	This study

PCR and oligonucleotides

All DNA amplifications by PCR were carried out with *Pfu* DNA polymerase in a gradient thermocycler (CA, Bio-Rad). PCR products were purified with QIAquick PCR Purification Kit (Qiagen). All oligonucleotide primers used in this study were synthesized from Sigma-Aldrich (Bangalore, India), which are listed in Table 3.

Gene synthesis and DNA sequencing

The gene sequence of superfolder green fluorescence protein (*sf*GFP) used in this study was synthesized from GenScript (NJ, USA). The synthesized gene was received as a recombinant plasmid in a cloning vector. The DNA sequences of all recombinants constructed in this study were confirmed by DNA sequencing Sigma-Aldrich (Bangalore, India).

Plasmid compatibility and stability test

Stability and compatibility of plasmids were studied as per the method described by Walia et al. 2007. *C. acetoacidophilum* harboring either single or two plasmid(s) were inoculated from a single colony into BHI medium containing appropriate antibiotics and grown overnight at 30 °C on a rotary shaker (200–

Table 2 List of plasmids used in this study

Plasmid	Size (kb)	Description	Reference
pGP1–2	7.2	Km^r, T7 RNA polymerase gene, p15A	Tabor and Richardson 1985
pEPR1[a]	7.3	Km^r, pCG1 replicon of *C. glutamicum*	Knoppova et al. 2007
pBK2	5.9	Km^r, pBL1 replicon of *C. glutamicum*	Mukherjee et al. 1990
pET28a	5.4	Km^r, ColE1, T7 expression cassette	Novagen
pACYC184	4.3	Cm^r, p15A, *E. coli* cloning vector	New England Biolabs
pJE1	2.7	Km^r, p15A, small *E. coli* cloning vector	This study
pJE-RNAP	5.3	Km^r, p15A, promoterless T7 RNA polymerase	This study
pJE-*fts*RNAP	5.8	Km^r, p15A, T7 RNA polymerase under P-ftsZ	This study
pJE-*k*RNAP	5.8	Km^r, p15A, T7 RNA polymerase under P-kan	This study
pJEC-*fts*RNAP	8.6	Km^r, p15A, pCG1, T7 RNA polymerase under P-ftsZ	This study
pJEC-*k*RNAP	8.6	Km^r, p15A, pCG1, T7 RNA polymerase under P-kan	This study
pBJ1	7.0	Km^r, ColE1, pBL1, T7 expression cassette	This study
pBJ2	8.0	Km^r, Cm^r, ColE1, pBL1, T7 expression cassette	This study
pBJ3	5.7	Cm^r, ColE1, pBL1, T7 expression cassette	This study
pBJ4	5.7	Cm^r, ColE1, pBL1, T7 expression cassette	This study
pBJ4-*sf*GFP	6.4	Cm^r, ColE1, pBL1, *sf*GFP under P-T7	This study

[a] Kindly gifted by Dr. Nesvera

Table 3 List of oligonucleotide primers used in this study

Primer	Sequence [5′→3′]	R. E. site
mut-F	TTCGCCCCCGTTTTCACTATGGGCAAATATTATACG	–
mut-R	GTATAATATTTGCCCATAGTGAAAACGGGGGCGAAG	–
JE1-F	GAATTCGGATCCTCCGGCGTTCAG	*Eco*RI, *Bam*HI
JE1-R	CTGCAGAATTTAAAAGGATCTAGG	*Pst*I
RNAP-F	CGAATTC**ATG**AACACGA	*Eco*RI
RNAP-R	GGGATCCGGAGTCGTATTG	*Bam*HI
PKAN-F	AATCTGCAGGGTCTGCCTCGTGAAG	*Pst*I
PKAN-R	GGTGAATTCTGTATTACTGTTTATG	*Eco*RI
PFTSZ-F	AATCTGCAGAGAAGTGCTTCCTGCG	*Pst*I
PFTSZ-R	AATGAATTCCTCGCCTTTCGAAGAGTTG	*Eco*RI
*sf*GFP-F	CGCGCCATGG**ATG**ACATCAACACCAC	*Nco*I
*sf*GFP-R	CGGTCGACTCACAATGTCGATGATGC	*Sal*I

Start codon (ATG) is in bold and restriction enzyme sites are underlined

250 rpm). Next day, 100 ml fresh BHI media without antibiotic was inoculated with 100 µl of overnight grown *C. acetoacidophilum* culture and was allowed to grow for 24 h until the cells reached stationary phase. An appropriate diluted culture was plated on BHI plates without antibiotics. Percentage of plasmid-harboring cells in culture was estimated by cross-counting of number of streaked colonies that appeared after transferring approximately 100 colonies on BHI plates with appropriate antibiotics. Roughly, an overnight grown culture were assumed to have cells of 10th generation; thus, the process of sub-culturing followed by plating without antibiotic and cross-counting streaked colonies on antibiotic plates were continued for 6 days to collect data of 60th generation.

Fluorescence measurements in *E. coli* and *C. acetoacidophilum*

The *sf*GFP, a robustly folded variant of GFP which has more intense fluorescence (Pédelacq et al. 2006), was used as a reporter gene to investigate and validate the auto-induced expression in newly constructed vector system(s) in *E. coli* and *C. acetoacidophilum*. The excitation and emission maxima of *sf*GFP are 488 nm and 515 nm, respectively. To measure the fluorescence intensity, 1 ml each of growing culture of *E. coli* and *C. acetoacidophilum* (in triplicate) were collected at different cultivation time points until the culture reached stationary phase and harvested down at 4,000 rpm, 5 min. Pellets were resuspended into the same volume of PBS and washed thrice with same buffer. The absorbance of washed samples was measured in a UV–vis spectrophotometer (Eppendorf, Germany) at OD_{600}, and subsequently the cells were adjusted to an OD_{600} of 0.5. The in vivo green fluorescence was measured with a fluorescence spectrometer set at an excitation wavelength of 480 nm and emission detection from 500 to 550 nm. The excitation slit was set at 7.5 and the emission slit at 10 nm. Documentation of the spectra was performed using fluorescence data manager software (Perkin Elmer, USA).

Fluorescence microscopy

Growing culture of *E. coli* or *C. acetoacidophilum* (1–2 ml) was pelleted down at 5,000 rpm. The pellet was subsequently washed four times with HEPES buffer and it was finally resuspended into an appropriate volume of the same buffer. A small volume of cells in HEPES buffer was fixed on a polylysine pre-treated slide. Microscopy was carried out using a fluorescence microscope (Olympus, USA) with a halogen light source attached to a high-definition camera, and image acquisition software (Andor, UK).

SDS–PAGE analysis

After normalization of cells, total proteins of *C. acetoacidophilum* cell lysate were separated on 12 %–15 % SDS–PAGE following standard method (Laemmli 1970) which was stained with Coomassie Brilliant Blue R-250 dye.

Results

A T7 RNA polymerase/promoter coupled dual plasmid system was constructed for both gram-negative *E. coli* and gram-positive *C. acetoacidophilum*, where one plasmid carries a T7 RNA polymerase gene and another one carries a strong T7 promoter. Two well-characterized corynebacterial replicons, pBL1 replicon of *Brevibacterium lactofermentum* (Santamaria et al. 1984) and pCG1 replicon of *C. glutamicum* (Ozaki et al. 1984), were selected for the present study to

develop two plasmid-based expression systems in *C. acetoacidophilum*.

Construction of plasmids carrying T7 RNA polymerase gene

Plasmid pGP1-2 (Tabor and Richardson 1985) was used as source of T7 RNA polymerase gene to construct T7 RNA polymerase-based plasmids. The 2.6-kb *Pst*I–*Bam*HI fragment of pGP1–2 consists of p15A origin of replication and kanamycin resistant gene (Km^r) cassette was PCR amplified using primers JE1-F and JE1-R. Also, a unique restriction site *Eco*RI was introduced into primer JE1-F just upstream to *Bam*HI restriction site to be used for further cloning site. The Pfu DNA polymerase amplified PCR product was self-ligated with T4 DNA polymerase, and the ligation mixture was subsequently transformed into *E. coli* Top10 competent cells. The transformed colonies were selected on LA-containing kanamycin plates. Thus, an *E. coli* replicating small plasmid pJE1 was obtained which contained three adjacent cloning sites *Bam*HI, *Eco*RI, and *Pst*I (Fig. 1a, b). In the next step, only coding sequence of T7 RNA polymerase gene (gene 1 of T7) was PCR amplified from the same plasmid pGP1–2 using primers RNAP-F and RNAP-R. The PCR product was subsequently cloned into plasmid pJE1 in between *Bam*HI and *Eco*RI restriction sites and so the obtained recombinant plasmid carrying promoterless T7 RNA polymerase gene was designated as pJE-RNAP (Fig. 1c). The orientation of T7 RNA polymerase and Km^r genes in pJE-RNAP is the same as that of plasmid pGP1–2, i.e., facing each other so that the strong terminator sequence of Km^r falls downstream to promoterless T7 RNA polymerase gene. Later, in order to supply functional promoter(s) to promoterless T7 RNA polymerase gene, promoter(s) of *filamentous temperature sensitive mutant* Z (*fts*Z) of *C. acetoacidophilum* and promoter of kanamycin resistant gene were separately cloned into pJE-RNAP

C. glutamicum and related genera viz. *Mycobacterium tuberculosis* are known to possess multiple promoters for their corresponding *fts*Z gene, present as a cluster within 500 bp upstream *fts*Z start codon (Letek et al. 2007; Kiran et al. 2009); therefore, about 500 bp upstream promoter region of *fts*Z was fished out from *C. acetoacidophilum* genome by PCR, using sequence-specific primers PFTSZ-F and PFTSZ-R, and the PCR amplified *fts*Z promoter regions were cloned in between *Pst*I and *Eco*RI restriction sites of plasmid pJE-RNAP. This resultant recombinant plasmid was designated as pJE-*fts*RNAP (Fig. 1d). In another construct, 400 bp upstream promoter region of Km^r gene was PCR amplified from plasmid pGP1–2 using primers PKAN-F and PKAN-R, and was cloned into pJE-RNAP at *Pst*I–*Eco*RI, and this recombinant plasmid was designated as pJE-*k*RNAP (Fig. 1d). Thus, we obtained two *E. coli* replicating plasmids harboring T7 RNA polymerase gene; in the first one the T7 RNA polymerase gene is under the control of multiple *fts*Z promoter of *C. acetoacidophilum*, whereas in the second one the same is under the control of a constitutive promoter, the Tn9 promoter of Km^r gene.

Both plasmids pJE-*k*RNAP and pJE-*fts*RNAP were further manipulated into shuttle plasmids to be used for *C. acetoacidophilum*. The 2.8-kb pCG1 replicon of corynebacteria was released from pEPR1 (Knoppova et al. 2007) by *Nru*I digestion and was subsequently cloned at dispensable *Dra*I site of pJE-*fts*RNAP and pJE-*k*RNAP, and so obtained *E. coli*–*Corynebacterium* sp. shuttle plasmids were designated as pJEC-*fts*RNAP and pJEC-*k*RNAP, respectively (Fig. 1e, f).

Construction of T7 promoter-based shuttle expression vectors

Plasmids based on pBL1 and pCG1 replicons replicate in *Corynebacterium* sp. by rolling circle mode but having independent requirements for their replication (Fernandez-Gonzalez et al. 1994; Archer and Sinskey 1993) and, therefore, are supposed to be compatible in corynebacterial cells. Since pCG1 replicon had been used for constructing plasmids carrying the T7 RNA polymerase gene, the pBL1 replicon was used here to construct T7 promoter-based shuttle plasmids.

The intact pBL1 replicon of corynebacteria was released from plasmid pBK2 (Mukherjee et al. 1990) by *Hinc*II digestion as a 2.7-kb fragment, and it was subsequently ligated with 4.2 kb *Pvu*II–*Pvu*II fragment of pET28a which consists of ColE1 origin of replication, kanamycin resistant (Km^r) gene cassette, and T7 expression region. The resultant corynebacteria–*E. coli* shuttle vector was designated as pBJ1 (Fig. 2a–c). Since the plasmids harboring T7 RNA polymerase carried the same Km^r gene cassette, which had to be co-transformed in same host cell, thus another antibiotic resistant gene cassette was needed for T7 promoter carrying plasmid. Therefore, 1 kb intact chloramphenicol resistant (Cm^r) gene cassette was excised from plasmid pACYC184 (Rose 1988) by *Sau*3AI digestion and subsequently cloned into pBJ1 at dispensable *Bgl*II site located upstream to T7 promoter region, and so obtained 8-kb shuttle plasmid was designated as pBJ2 (Fig. 2c–e). The Cm^r gene is known to be functional in both *E. coli* and corynebacteria (Rose 1988; Eikmanns et al. 1991). Plasmid pBJ2 carried both Km^r gene cassette and Cm^r gene cassette, and its size was too large to be an ideal expression vector; thus, plasmid pBJ2 was trimmed into a 5.7-kb shuttle plasmid, pBJ3, possessing only the Cm^r marker. To obtain plasmid pBJ3, plasmid pBJ2 was digested with *Hae*II, and a 1.5-kb fragment, carrying both T7 expression cassette and Cm^r marker, was excised out and subsequently their ends were blunted with T4 DNA polymerase. In a separate

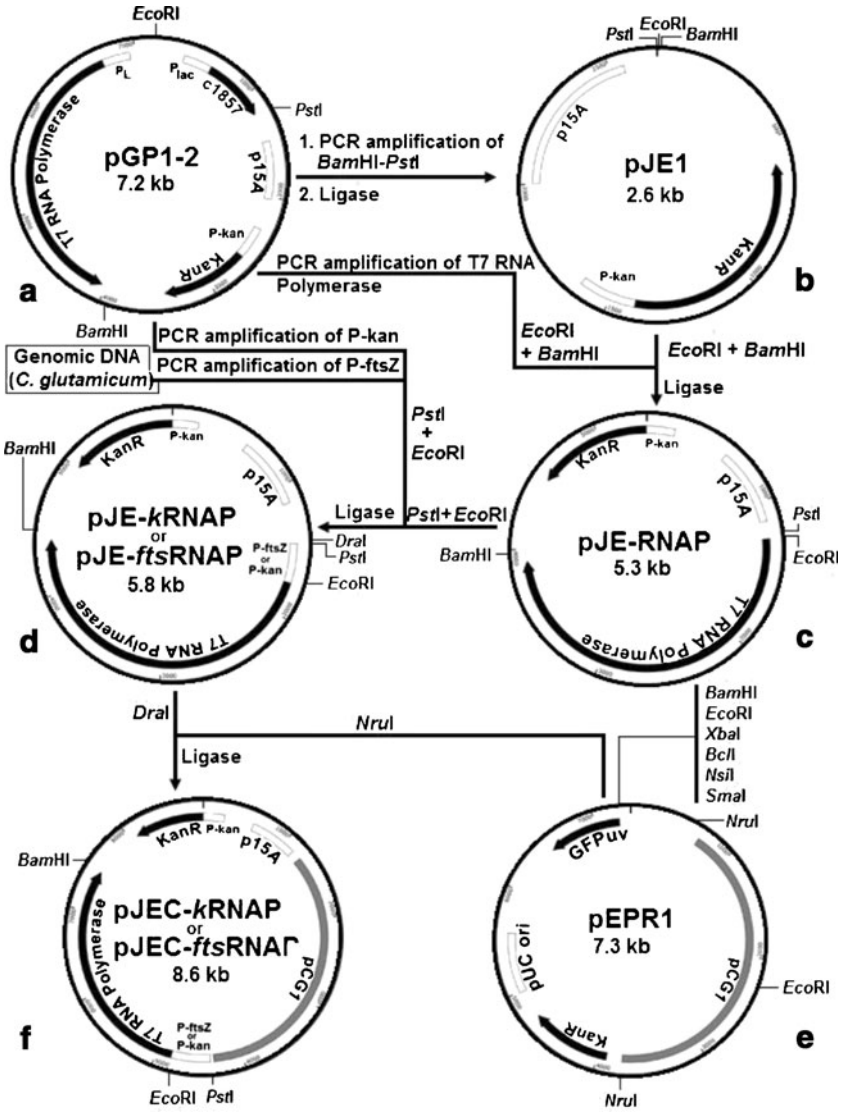

Fig. 1 Construction of plasmids with T7 RNA polymerase gene for *E. coli* and *Corynebacterium* sp. **a** *E. coli* replicating plasmid pGP1–2 used as sources of T7 RNA polymerase gene, p15 ori and kanamycin resistant gene cassette, Km^r, as well as promoter of kanamycin resistance gene, P-kan; **b** plasmid pJE1 carrying *E. coli* ori, p15A, and kanamycin resistance gene cassette, Km^r; **c** promoterless T7 RNA polymerase harboring plasmid pJE-RNAP achieved after cloning of only coding sequences of T7 RNA polymerase gene into pJE1; **d** plasmids pJE-*fts*RNAP and pJE-*k*RNAP achieved after cloning of promoters of *fts*Z and Km^r, respectively, into plasmid pJE-RNAP upstream to T7 RNA polymerase gene; **e** plasmid pEPR1 used as a source of pCG1 replicon; **f** *E. coli–Corynebacterium* sp. shuttle plasmids pJEC-*fts*RNAP and pJEC-*k*RNAP achieved after cloning of pCG1 replicon into pJE-*fts*RNAP and pJE-*k*RNAP, respectively

restriction digestion, the 4.2-kb fragment was released from pBJ2 by *Nru*I and *Eco*RV double digestion. This fragment, carrying both pBL1 replicon of corynebacteria and ColE1 replicon of *E. coli*, was subsequently ligated with 1.5 kb *Hae*II-blunt fragment, carrying both T7 expression region and Cm^r gene cassette, and thus the shuttle plasmid pBJ3 was obtained (Fig. 2e–g). Since the *Nco*I site of MCS is no longer available for cloning due to another *Nco*I site that falls into Cm^r gene, therefore, the *Nco*I recognition site of the Cm^r gene was modified through PCR-based site-directed mutagenesis using primers mut-F and mut-R, and thus obtained final construct was termed as pBJ4 (Fig. 2g, h). The commercially available *E. coli* T7 plasmid pET28a (Novagen) has both N- and C-terminal 6× His tags to facilitate Ni–NTA affinity purification of recombinant proteins. The Ni–NTA affinity chromatography-based purification does not depend on the host chosen for recombinant protein expression; rather, its only requirement is that the recombinant protein should have a His tag (Crowe et al. 1994). Because the T7 expression cassettes of *E. coli–Corynebacterium* sp. T7 shuttle plasmid pBJ4 is derived from pET28a, therefore if desirable, both N- or C-terminal His-tagged recombinant proteins can be expressed through plasmid pBJ4 and can subsequently be purified by Ni–NTA affinity chromatography.

Corynebacterial replicons pBL1 and pCG1 are compatible in *C. acetoacidophilum* and thus suited for dual plasmid system

Compatibility and co-stability of BL1 and pCG1 replicons were tested in *C. acetoacidophilum* using recombinant plasmids JEC-*fts*RNAP (pCG1 replicon) and pBJ4 (pBL1 replicon). Both plasmids were sequentially co-transformed into *C. acetoacidophilum* through electroporation, and final transformants were selected on both kanamycin- and chloramphenicol-containing BHI plates. Both plasmids were found to be stable in corynebacteria for up to 60

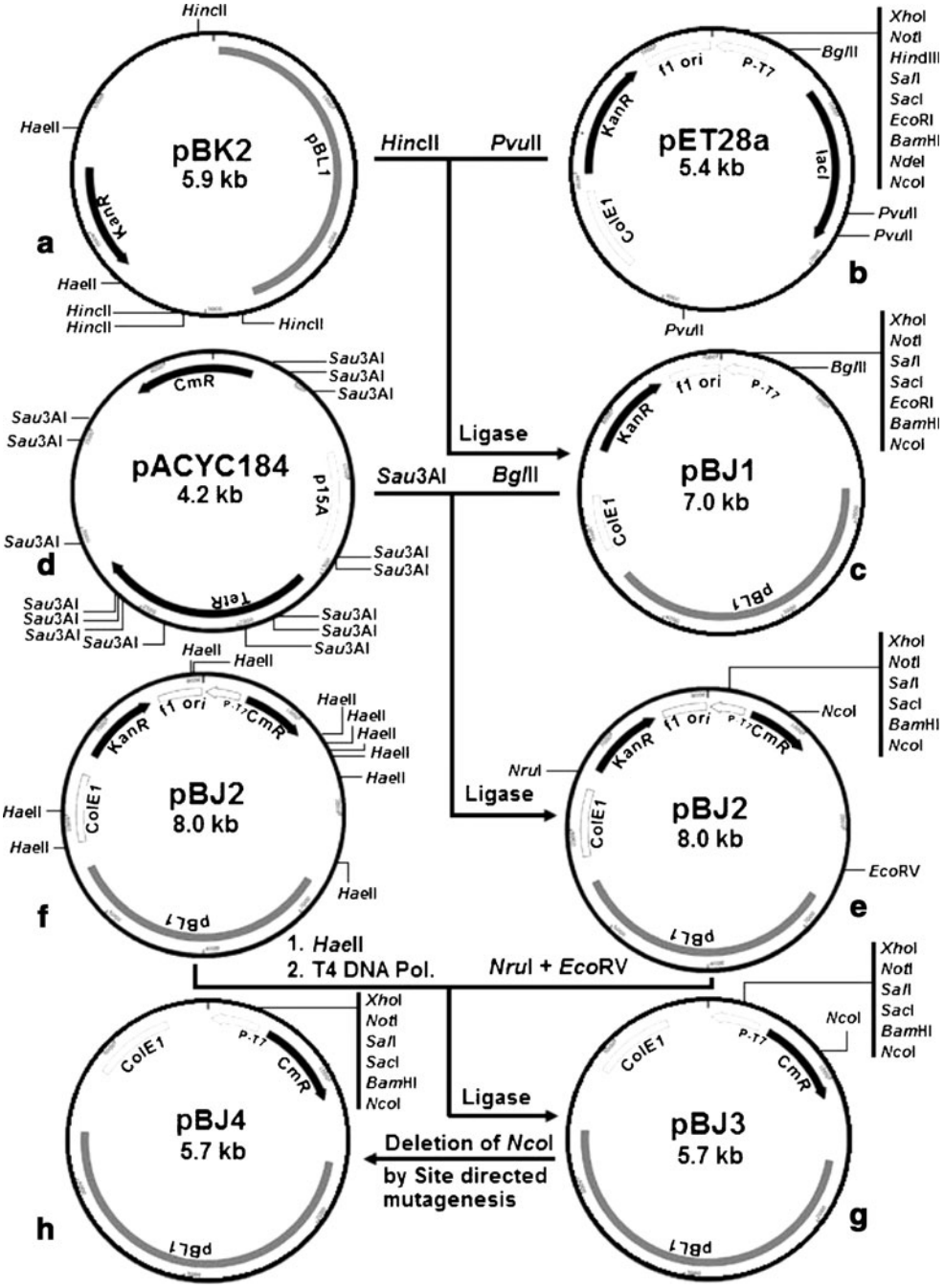

Fig. 2 Construction of T7 promoter-based *E. coli*–*Corynebacterium* sp. shuttle expression plasmids. **a** Corynebacterial plasmid pBK2 used as a source of corynebacterial replicon, pBL1; **b** plasmid pET28a, a T7 promoter-based expression vector for *E. coli*; **c** *E. coli*–*Corynebacterium* sp. T7 shuttle plasmid pBJ1 derived from pET28a and pBK2, carrying Km^r gene cassette; **d** plasmid pACYC184, a source of Cm^r gene cassette; **e** *E. coli*–corynebacteria T7 shuttle plasmid pBJ2 derived from pBJ1 and pACYC184, Km^r and Cm^r; **f** plasmid pBJ2; **g** *E. coli*–*Corynebacterium* sp. T7 shuttle plasmid pBJ3 derived after trimming non-essential regions of plasmid pBJ2; **h** *E. coli*–*Corynebacterium* sp. T7 shuttle plasmid pBJ4 derived after site-directed mutagenesis of plasmid pBJ3 at *Nco*I site of Cm^r gene

generations without any selection pressure. Stability of both plasmids in *C. acetoacidophilum* was further confirmed by plasmid isolation at the end of a batch cultivation (Fig. S1).

High-level auto-induced expression of *sf*GFP through T7 promoter in *E. coli*

The intact coding sequence of *sf*GFP was PCR amplified from synthetic construct pUC–*sf*GFP using primers SFGFP-F and SFGFP-R, and was subsequently cloned under strong T7 promoter of pBJ4 at *Nco*I–*Sal*I sites to obtain pBJ4-*sf*GFP. The recombinant plasmid was transformed into routinely used T7 polymerase host *E. coli* BL21 (DE3) along with Top10 (*k*RNAP) and Top10 (*fts*RNAP) expression hosts.

In Top10 (*k*RNAP) and Top10 (*fts*RNAP) expression hosts, the T7 RNA polymerase gene was supplied through low copy number plasmids pJE-*k*RNAP and pJE-*fts*RNAP, respectively. In Top10 (*k*RNAP), T7 RNA polymerase gene is under the control of a constitutive promoter (P-kan) of Tn9-derived Km^r resistant gene, whereas in Top10 (*fts*RNAP) the same is under control of the

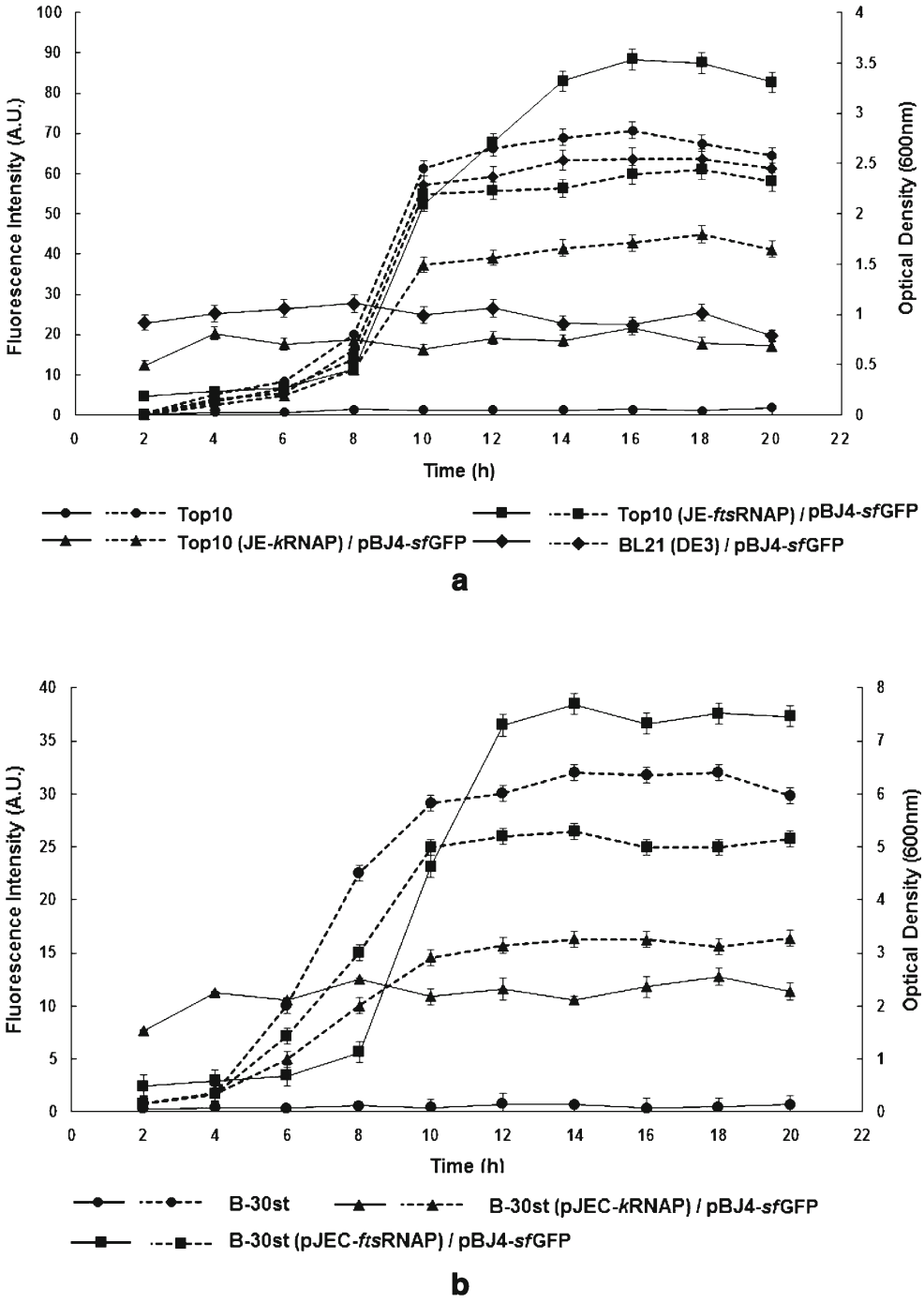

Fig. 3 Comparative expression of *sf*GFP into *E. coli* and *C. acetoacidophilum* at different growth phases. **a** Relative fluorescence intensities (*smooth lines*) and their corresponding optical densities, OD_{600nm} (*dotted lines*), for *E. coli* hosts either lacking T7 RNA polymerase gene (Top10/pBJ4-*sf*GFP) or harboring T7 RNA polymerase under *fts*Z promoter(s) [Top10 (pJE-*fts*RNAP)/pBJ4-*sf*GFP], or constitutive Km^r promoter [Top10 (pJE-*k*RNAP)/pBJ4-*sf*GFP], or constitutive lacUV5 promoter [BL21 (DE3)/pBJ4-*sf*GFP]. LacUV5 promoter of BL21 (DE3) was made constitutive by adding 1 mM IPTG at the time of inoculation. Untransformed *E. coli* Top10 were taken as negative control. **b** Relative fluorescence intensities (*smooth lines*) and their corresponding optical densities, OD_{600nm} (*dotted lines*), for *C. acetoacidophilum* hosts either lacking T7 RNA polymerase gene (B-30st) or harboring T7 RNA polymerase under *fts*Z promoter(s) [B-30st (pJEC-*fts*RNAP)/pBJ4-*sf*GFP], or constitutive Km^r promoter [B-30st (pJEC-*k*RNAP)/pBJ4-*sf*GFP]. Untransformed *C. acetoacidophilum* B-30st were taken as negative control

*fts*Z promoter (P-*fts*Z) of *C. acetoacidophilum*. In BL21 (DE3), chromosomally integrated single copy of T7 RNA polymerase gene is under control of the IPTG-inducible lacUV5 promoter which was made constitutive by adding a saturating concentration of IPTG (1 mM) into medium at the time of inoculation.

Our results show that in Top10 (*fts*RNAP) host, *sf*GFP expression is too low during initial growth phase, but as the growth of culture proceeds towards late-log phase and stationary phase, the expression level of *sf*GFP dramatically increases. Notably, the expression level of *sf*GFP at stationary phase of Top10 (*fts*RNAP) host was much higher than that of Top10 (*k*RNAP) and BL21 (DE3) hosts which were constitutively expressing T7 RNA polymerase (Fig. 3a). Transformation of *E. coli* Top10 (*fts*RNAP) cells with plasmid pB4-*sf*GFP gave intense green colonies on antibiotics-containing LA plate (Fig. 4a). High level of *sf*GFP into Top10 (*fts*RNAP) cells was also evident from fluorescence microscopy (Fig. 4b).

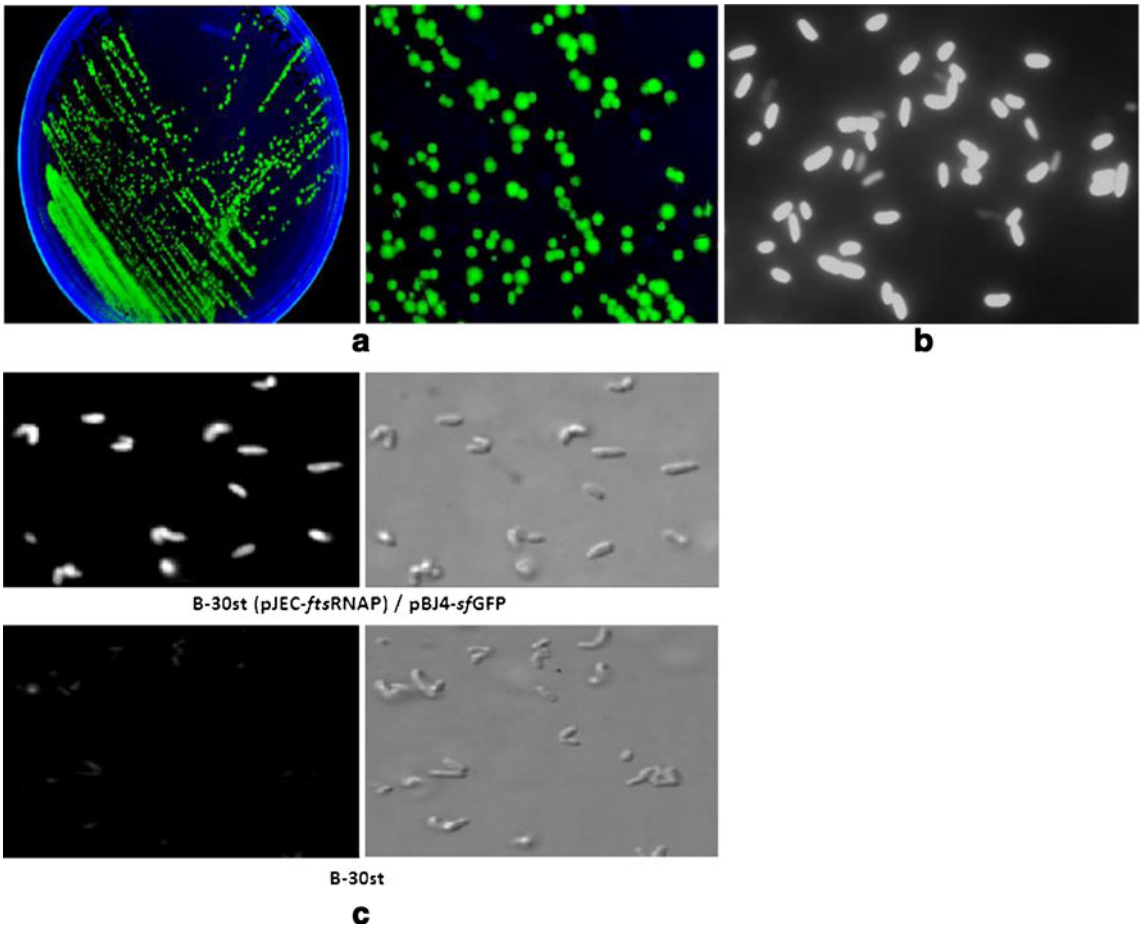

Fig. 4 T7 promoter-derived expression of *sf*GFP into *E. coli* Top10 and corynebacteria co-transformed with plasmids pJE-*fts*RNAP and pBJ4-*sf*GFP. **a** Green *E. coli* colonies on Luria-agar plates viewed under trans-UV as a result of *sf*GFP expression; **b** fluorescence microscopy image of *E. coli* cells expressing *sf*GFP; **c** fluorescence microscopy images of *C. acetoacidophilum* (B-30st): expression of sfGFP in cells carrying plasmids pJEC-*fts*RNAP and pBJ4-*sf*GFP

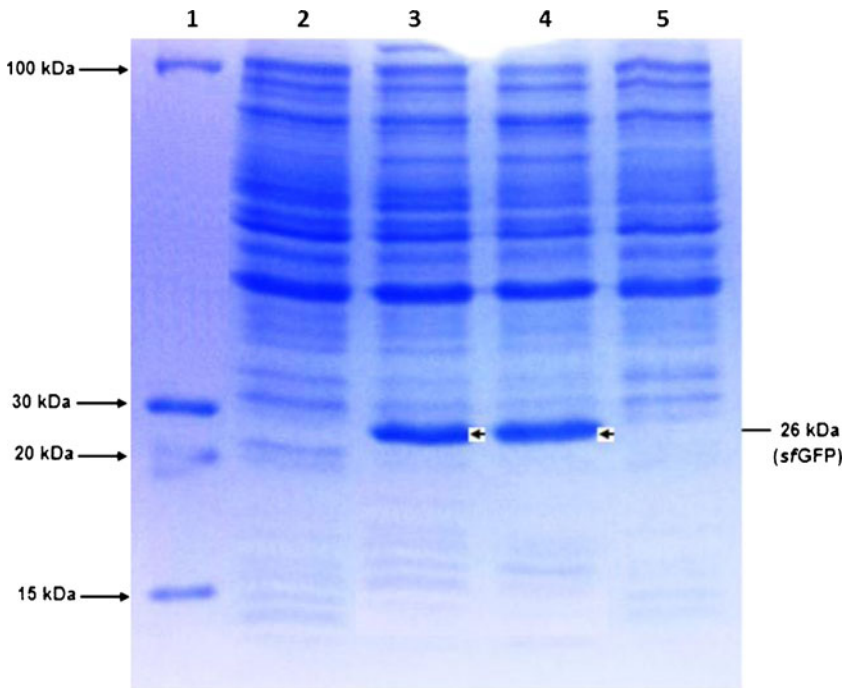

Fig. 5 SDS–PAGE analysis for expression of *sf*GFP into *C. acetoacidophilum*. *Lane-1* low molecular weight protein marker, *2–4* cell lysates of B-30st cells co-transformed with plasmids pJEC-*fts*RNAP and pBJ4-*sf*GFP after 2 h, 12 h, and 16 h growth, respectively; *5* cell lysate of B-30st without plasmid (negative control). An intense band of *sf*GFP (26 kDa) is visible in late-log and stationary phase samples in *lane 3* (12 h) and *lane 4* (16 h), respectively, which is undetectable in lag or very early log phase sample in *lane 2* (4 h)

T7 Promoter system is functional in *C. acetoacidophilum* in a growth-phase-dependent manner

To study the function of T7 RNA polymerase gene in *C. acetoacidophilum*, recombinant plasmid pBJ4-*sf*GFP was co-transformed into *C. acetoacidophilum* along with either pJEC-*k*RNAP or pJEC-*fts*RNAP by electroporation. Expression of *sf*GFP into *C. acetoacidophilum* was checked by measuring fluorescence intensity as earlier done for *E. coli*. Although, as compared to *E. coli*, in *C. acetoacidophilum* the expression level of *sf*GFP was found to be lesser, the two-plasmid-based T7 RNA polymerase-dependent system was also found to be functional in *C. acetoacidophilum*. Our results reveal that in *C. acetoacidophilum* host that was constitutively expressing T7 RNA polymerase, i.e., B.30st (pJEC-*k*RNAP), *sf*GFP expression level was almost constant throughout the growth phases (~10 A.U.), whereas the expression of recombinant protein (*sf*GFP) in *C. acetoacidophilum* host that was expressing T7 RNA polymerase through *fts*Z promoter(s), i.e., B-30st (pJEC-*fts*RNAP), was almost shut off up to a certain level of growth phase, and after crossing that level, the expression of recombinant protein through T7 promoter started increasing drastically without the addition of any external inducer. Eventually, the stationary phase expression level of *sf*GFP in B-30st (pJEC-*fts*RNAP) host was many folds higher than that of the constitutive one (Fig. 3b). Fluorescence microscopy and SDS–PAGE data further confirm high-level expression of *sf*GFP into *fts*Z promoter-dependent T7 RNA polymerase expressing *C. acetoacidophilum* host (Figs. 4c and 5).

Discussion

In prokaryotes, *fts*Z, a tubulin-like cell cycle protein, is conserved throughout the bacteria and the archaea. It polymerizes to make a contractile ring, the Z-ring, during cell division at the division site. The topology, the frequency, and the timing of division are solely regulated by the varying concentration of *fts*Z in host cell (Lutkenhaus and Addinall 1997). For *fts*Z transcription, multiple promoters have been elucidated in diverse bacteria (Letek et al. 2007; Kiran et al. 2009), and the overall expression level of *fts*Z is known to oscillate during cell cycle due to the cumulative activity of various promoters (Garrido et al. 1993).

The *fts*Z promoter activity is almost shut off in non-dividing cells and becomes active in the dividing ones since the *fts*Z protein plays a pivotal role in cell division. Moreover, the activity of *fts*Z promoter has been observed to be growth dependent. During lag and early log phases, where no significant cell division takes place, activity of *fts*Z is hardly detectable, but the promoter is highly active by an unknown mechanism when cells reach late-log and stationary phase (Weart and Levin 2003).

In *E. coli*, BL21 (DE3) is the most prevalent system for high-level recombinant protein production where chromosomally integrated T7 RNA polymerase is under the IPTG-inducible UV5 promoter (Studier and Moffatt 1986). Tabor and Richardson (1985) developed a dual-plasmid-based T7 system where the plasmid pGP1–2 harbors T7 RNA polymerase which is under the control of heat-inducible promoter P_L of lambda phage. Chao et al. (2002) developed *E. coli* BL21 (BAD) strain for toxic protein expression placing the chromosomally integrated T7 RNA polymerase gene under tightly regulated L-arabinose-inducible *ara*BAD promoter of *E. coli*. These T7-based systems in *E. coli* are proven essential protein expression tools, but they all are based on external inducers. While IPTG is expensive and imposes toxicity to the growing culture, heat induction may not be a preferred choice as it increases the protease level which may eventually degrade the target proteins. Also, heat may cause significant death of bacterial host cells. An expression system independent of external inducer is a cost-effective system and may be best suited for easy scale-up.

As far as corynebacteria are concerned, very little success is achieved so far with the attempts made to develop an expression system in corynebacteria, inducible and constitutive, capable of producing a high level of target proteins. Lack of *lac*Y and *ara*E in most of the *Corynebacterium* strains and impermeability of its cell membrane to common inducers, IPTG and L-arabinose, through diffusion process come as bottlenecks to develop an IPTG/L-arabinose-inducible system in *Corynebacterium* sp. Also, heterologous cloning of *lac*Y and *ara*E transporters into *C. glutamicum* did not produce desirable results (Zhang et al. 2012).

Expression systems based on strong T7 promoter are widely used for the industrial production of proteins where high-level expression of heterologous protein is of prime concern. In this context, this is the very first report of a T7 promoter-based system in *Corynebacterium* sp. Its function was shown in both *C. acetoacidophilum* and gram-negative *E. coli*. It might be also used for high-level production of heterologous proteins in *C. glutamicum* and other related corynebacteria.

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

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