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ORIGINAL PAPER

Enhancing functional expression of heterologous lipase B in *Escherichia coli* by extracellular secretion

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Abstract Functional expression of recombinant *Pseudozyma antarctica* lipase B (PalB) in *Escherichia coli* has been technically problematic due to protein misfolding, ineffective disulfide bond formation, and protein instability associated with intracellular proteolysis. To overcome these problems, an alternative approach was explored in this study by extracellular secretion of PalB via two Sec-independent secretion systems, i.e., the α-hemolysin (type I) and the modified flagellar (type III) secretion systems, which can export proteins of interest from the cytoplasm directly to the exterior of the cell. Both shaker flask and bioreactor cultivations were performed to characterize the developed PalB expression/secretion systems. Bioactive PalB was expressed and secreted extracellularly either as a HlyA fusion (i.e., PalB-HlyA via type I system) or an intact protein (via type III system). However, the secretion intermediates in the intracellular fraction of culture samples were non-bioactive even though they were soluble, suggesting that the extracellular secretion did mediate the development of PalB activity. Also importantly, the secretion strategy appeared to have a minimum impact on cell physiology. PalB secretion via the type I system was fast with higher specific PalB activities but poor cell growth. On the other hand, the secretion via the type III system was slow with lower specific PalB activities but effective cell growth.

Keywords *Escherichia coli* · Flagellum · Hemolysin · Lipase B · Protein secretion · *Pseudozyma antarctica* · Recombinant protein production

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Introduction

Escherichia coli retains its popularity as the most versatile host for recombinant protein production because of the many expression advantages [16, 29, 34]. Given the extensive knowledge associated with heterologous protein expression in *E. coli*, technical limitations are still encountered, particularly including posttranslational processing, protein misfolding and aggregation, proteolysis, and recombinant protein toxicity [28].

Cytoplasm is the intracellular compartment where all nascent proteins are first synthesized and most of them reside. However, it might not serve as a suitable destination for recombinant protein production because of the liability to protein proteolysis [42], the inability to form disulfide bonds [36], the tendency to protein misfolding [24], and the complicated downstream processing [41]. These drawbacks can be potentially overcome by secretion of foreign gene products to the periplasm or extracellular medium [31].

All the periplasmic and outer membrane proteins in *E. coli* require the secretion of their corresponding precursors across the cytoplasmic membrane. The major mechanism driving such translocation is the Sec-dependant type II secretion pathway, which is mediated by a cleavable signal peptide at the N terminus [10]. A wide variety of signal peptides, such as PelB [4], PhoA, OmpA [51], and SpA [33], have been adopted successfully as a genetic strategy to secrete recombinant proteins for expression in the periplasm or on the outer membrane of *E. coli*.

By contrast, very few *E. coli* proteins are known to be extracellularly secreted due to the presence of another secretion barrier of the outer membrane. The type I secretion system, known as the ABC transporter, secretes *E. coli* toxins and exoenzymes via a one-step process

across both the cytoplasmic and outer membranes without a periplasmic intermediate [5]. On the other hand, the type III secretion system represents a specific mechanism associated with the secretion of pathogenesis factors or flagellar proteins from the cytoplasm, bypassing the periplasm, directly to the exterior of the bacteria [44].

Despite the limited natural mechanisms for protein secretion in *E. coli*, genetic strategies have been developed to release recombinant proteins extracellularly, including (1) the use of leaky mutant host strains and cell wall-less L-form *E. coli* [19], (2) chemical or biochemical permeabilization of the outer membrane [10, 39], and (3) coexpression of protein release factors such as bacteriocin release protein (BRP) [48], colicin E1 lysis protein (Kil), *tolAIII*, and *out* genes [49]. However, these approaches on the basis of permeabilizing cells are limited to the release of periplasmic proteins (which are typically exported from the cytoplasm via type II secretion system) and often deteriorate the physiological condition of producing cells. Consequently, the recombinant protein yield is hampered. Type I or III secretion systems, therefore, appear to be an alternative by which extracellular production of proteins can be performed with minimum physiological impact. In fact, several proteins, which are otherwise hard to express intracellularly, have been functionally expressed and extracellularly secreted via the hemolysin (type I) system [7, 13, 47] and the flagellar (type III) system [27].

PalB, a lipase B from *Pseudozyma antarctica* (previously known as *Candida antarctica*), is an important industrial enzyme with a wide range of applications [3]. Herein, we are exploring another strategy for functional expression of recombinant PalB, namely extracellular secretion via type I and III systems (Fig. 1). Previously, PalB was expressed heterologously in fungi and yeasts, such as *Aspergillus oryzae* [21], *Pichia pastoris* [37], and *Saccharomyces cerevisiae* [57]. Given the popularity of *E. coli*, functional expression of PalB in this host was not demonstrated until recently [6, 25, 52–54]. Since there are three intramolecular disulfide bonds in PalB and their formation can potentially affect the development of protein structure and bioactivity, functional expression of PalB in the oxidative periplasm of *E. coli* appears to be a rational approach. However, heterologous PalB targeting in the periplasm was highly unstable and subjected to proteolysis [52]. Cytoplasmic expression of PalB in the reducing cytoplasm of wild-type *E. coli* suffered ineffective disulfide bond formation. Though this technical issue could be circumvented by the expression in the oxidative cytoplasm of genetically modified *E. coli* (i.e., Origami^{TM}), protein misfolding remained problematic [53].

Given the above limitations in disulfide bond formation and protein misfolding, extracellular secretion appears to be a plausible exploration. In this study, using the hemolysin secretion apparatus, PalB-HlyA protein fusion was extracellularly produced with relatively fast secretion and high PalB activities. On the other hand, though PalB secreted via the flagellar system is an intact protein, the secretion was not initiated until 24 h after cultivation. Both two protein secretion systems had a minimum physiological impact on producing cells.

Materials and methods

Bacterial strains and plasmids

Bacterial strains, plasmids, and oligonucleotides used in this study are summarized in Table 1 and are briefly described below. DH5α was used as the host strain for molecular cloning. BL21(DE3), DH5α, HB101, JM109, and MC4100 were used as the expression hosts for PalB secretion studies using the hemolysin transporter. MKS12 was used as the host strain for PalB secretion studies using the flagellar system.

Molecular cloning was performed according to standard protocols [38]. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Polymerase chain reaction (PCR) was conducted in an automated thermal cycler (GeneAmp Thermocycler, Applied Biosystems, Foster City, CA). Purification of plasmid DNA was performed using a spin-column kit purchased from Clontech (Palo Alto, CA). Plasmid transformation was carried out using an electroporator (*E. coli* Pulser, Bio-Rad, Hercules, CA) or a chemical method according to Chung and Miller [11].

The plasmid pEHLYAPalB was used for extracellular secretion of PalB-HlyA fusion via the hemolysin system. The *palB* gene was PCR-amplified with *Pfu* (Stratagene, La Jolla, CA) as the PCR polymerase, pPCRscriptPalB-L as the template, and P10 and pEHREV as the primers. The amplified DNA fragment was digested with *Nco*I/*Eag*I and ligated with similarly digested pEHLYA2-SD to form pEHLYAPalB. The plasmid pVDL9.3 was used for the production of HlyB and HlyD. The plasmid p5′3′UTRPalB was used for extracellular secretion of PalB via the *E. coli* flagellar system. The *Pvu*I/*Sac*I fragment of p5′UTR was subcloned into pMCS3′UTR to form p5′3′UTR containing the 173-bp 5′- $fliC_{MG1655}$ promoter sequence and the 321-bp 3′- untranslated region (UTR) of the $fliC_{MG1655}$ gene. The *palB* gene was PCR-amplified with *Pfu* as the PCR polymerase, pPCRscript-PalB-L as the template, and P10 and P22 as the primers. The amplified DNA fragment was digested with *Nco*I/*Sac*I and ligated to similarly digested p5′3′UTR to form p5′3′UTRPalB.

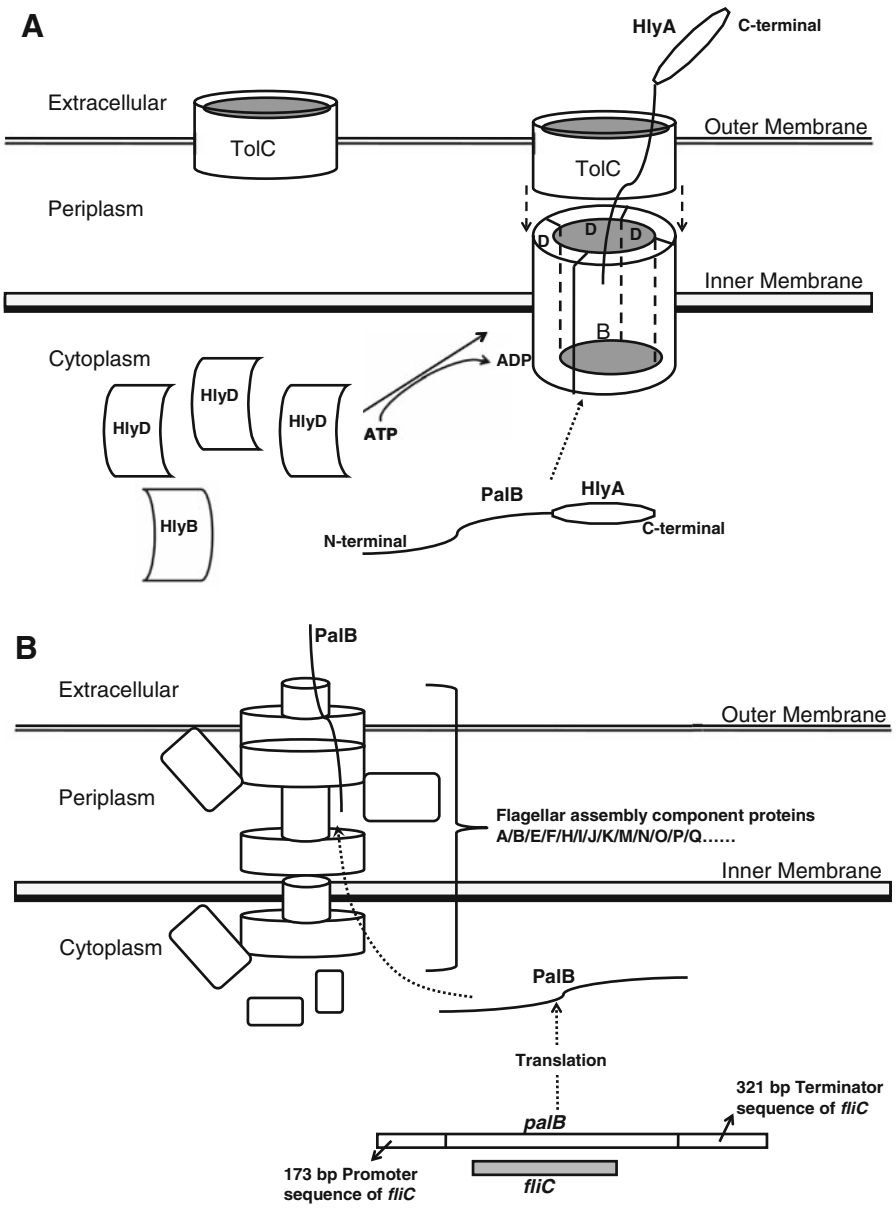

Fig. 1 Extracellular secretion of heterologous PalB in *E. coli* via type I and type III secretion systems. **a** The hemolysin (type I) secretion system belongs to the ABC transporter family, which recognizes the C-terminal amino acids of hemolysin toxin HlyA for protein secretion without requiring an N-terminal signal peptide [17]. The inner membrane proteins, i.e., HlyB and HlyD trimer, form an assembly with concurrent ATP hydrolysis [13, 15] to interact with the outer membrane protein TolC [23]. A hydrophilic channel connecting the two membranes forms an export conduit through which the recombinant PalB fused to the 23 kDa C-terminal of HlyA is co-exported extracellularly. **b** The *E. coli* flagellar (type III) secretion system can secrete the filamentous flagellar protein (FliC) through the interaction with various flagellar assembly component proteins [32]. In order to conduct recombinant protein secretion using this system, the flagellar protein gene *fliC* is replaced by the target gene of interest (*palB* in this study) flanked by the untranslated regions (UTR) of the *fliC* gene, i.e., the 173-bp promoter sequence of $fliC_{MG1655}$ at the 5′ end and 321-bp terminator sequence of $fliC_{MG1655}$ at the 3′ end [27]. The exact nature of the secretion signals is still unknown. One model suggests that the secretion could be cotranslational using the 5′UTR region of mRNA as the signal [2], whereas another model describes the secretion as posttranslational using a protein sequence as a signal [26]

Cultivation

Cells were revived by streaking the stock culture stored at −80°C on an LB agar plate (5 g/l NaCl, 5 g/l Bacto yeast extract, 10 g/l Bacto tryptone, and 15 g/l Bacto agar). The plate was incubated at 37°C for approximately 15 h. An isolated single colony was picked to inoculate 25 ml of LB medium, which was then incubated at 37°C in a rotary shaker at 200 rpm for approximately 15 h. The medium was supplemented with 50 μg/ml ampicillin

Table 1 Strains, plasmids and oligonucleotides

Strain	Relevant genotype	Source and reference
BL21(DE3)	F^- *ompT dcm lon hsdS*$_B$ (r_B^-, m_B^-) *gal* λ(DE3[*lacI ind1 sam7 nin5 lacUV5-T7* gene 1])	[35]
DH5α	F′/*(Φ80 dlac Δ(lacZ)M15) Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44 lambda-thi-1 gyrA96 relA1*	[50]
HB101	F^- *hsdS20 leuB6 recA13 ara-14 proA2 lacY1 thi-1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	[8]
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1 mcrA* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	[55]
MC4100	*araD 139*Δ(*argF-lac*)*U169 rpsL150 relA1 flb3501 deoC ptsF25 rbsR*	[9]
MKS12	Deletion of 1,407 bp fliD gene and 1,497 bp fliC gene of *E. coli* MG1655 Δ*fimA-H*[18]	[27]
Plasmid		
p5′UTR	173 bp promoter sequence of *fliC*$_{MG1655}$ in *Pvu*I-*Sca*I site of pBR322, additional *Nco*I-*Sac*I sites preceding *Sca*I, Tc^R	[27]
pMCS3′UTR	321 bp 3′UTR DNA sequence of *fliC*$_{MG1655}$ in *Sca*I-*EcoR*I site of pBR322, Tc^R	[27]
p5′3′UTR	173 bp promoter sequence of *fliC*$_{MG1655}$ in *Pvu*I-*Sca*I site of pMCS3′UTR, additional *Nco*I-*Sac*I sites preceding *Sca*I, Tc^R	This study
p5′3′UTRPalB	5′UTR::*palB*::3′UTR, Ori (pBR322), Tc^R	This study
pEHLYA2-SD	P_{lac}::HlyA, Ap^R	[13]
pEHLYAPalB	P_{lac}::*palB*::*hlyA*, Ap^R	This study
pPCRscriptPalB-L	PCR cloning of *palB*, Ap^R	This lab
pVDL9.3	P_{lac}::*hlyB*; *hlyD*, Cm^R	[13]
Oligonucleotides		
P10	5′-GG*CCATGG*GTCTACCTTCCGGTTCGG-3′	[54]
P22	5′-CT*GAGCTC*TCAGGGGGTGACGATGCCGGAG-3′	[54]
pEHREV	5′-ACGGAGCTCGAATTC*GGCCG*GGGTGACGAT-3′	This study

**Italic* mutation nucleotides, *underline* restriction site

(Ap), 34 μg/ml chloramphenicol (Cm), or 10 μg/ml tetracycline (Tc) when necessary. Erlenmeyer flasks containing 25-ml LB medium were inoculated with the seed culture and were shaken in a rotary shaker at 28 or 37°C and 200 rpm. For the hemolysin secretion system, the culture was supplemented with isopropyl β-D-thiogalactopyranoside (IPTG) for induction when the cell density reached approximately 0.5 OD_{600}. After induction, the Erlenmeyer flasks were further shaken, and samples were collected at appropriate times. All the shaker-flask cultivations were conducted at least in duplicate. For bioreactor cultivations, the above-described seed culture was used to inoculate a bench-top bioreactor (Omni-Culture, VirTis, Gardiner, NY) containing 1-l working volume of LB medium with 10 μl/l Antifoam 289 (Sigma, St. Louis, MO). IPTG at 0.5 mM was added for induction purposes when the culture cell density reached ~0.5 OD_{600}. The bioreactor was purged with filter-sterilized air at 2 l/min for aeration. The culture pH was regulated at 7.0 ± 0.1 by adding 3 N NH_4OH or 3 N HCl using a combination of a pH electrode (Mettler-Toledo, Switzerland), a pH controller (PC310, Suntex, Taipei, Taiwan), and two peristaltic pumps (101U/R, Watson Marlow, Falmouth, UK). The bioreactor was operated at 28 or 37°C and 650 rpm.

Analytical methods

The culture sample was appropriately diluted with saline solution for measuring cell density in OD_{600} with a spectrophotometer (DU®520, Beckman Coulter, Fullerton, CA). For the preparation of cell extract, cells at an amount of 20 OD_{600} units (defined as 'OD_{600} × ml') were centrifuged at 2°C and 6,000*g* for 6 min [52–54]. The supernatant containing extracellular proteins was analyzed for secreted PalB. The cell pellet was resuspended in 0.75 ml of sodium phosphate buffer (0.05 M, pH 7.5). The cell suspension was sonicated for 4 min using an ultrasonic processor (Misonix, Farmingdale, NY) and then centrifuged at 4°C and 12,000*g* for 15 min. The supernatant containing soluble proteins was assayed for the intracellular PalB activity. The pellet containing insoluble proteins and cell debris was washed with phosphate buffer, resuspended in TE/SDS buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, 1% SDS), and heated to 100°C for 5 min. The protein content of the pellet was analyzed as the insoluble fraction (data not shown).

PalB activity was qualitatively evaluated by the area and transparency of the halo formed on tributyrin agar plates. To conduct this, the soluble fractions of 50-μl cell lysates were loaded on the plate incubated at 37°C overnight.

On the other hand, PalB enzyme assay was conducted using a pH stat (Brinkman Metrohm 842 Titrando, Riverview, FL). An appropriate volume of the extracellular or soluble cell lysate fraction was added to 5 ml of 2% tributyrin emulsion in water at 37°C, and the reaction solution was maintained at pH 8.0 by controlled addition of 0.02 N NaOH [45]. One unit of enzyme activity is defined as the amount of enzyme required to liberate one μmole of fatty acid per min.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a Mini-PROTEIN® III electrophoresis cell (Bio-Rad) using a 12.5% polyacrylamide separating gel stacked with a 4% polyacrylamide stacking gel. Protein samples of 0.02 OD_{600} units for extracellular fraction, 0.017 OD_{600} units for the soluble intracellular fraction, and 0.2 OD_{600} units for insoluble intracellular fractions (data not shown), respectively were used. Electrophoresis was conducted under a constant voltage of 200 V for 45 min. To conduct Western blotting, proteins on the polyacrylamide gel were electroblotted to a polyvinylidene difluoride membrane using a Mini Trans-Blot cell (Bio-Rad) according to a standard protocol [46]. The electrophoretic transfer was conducted at a constant voltage of 100 V for 1 h. Primary anti-PalB antibodies were raised in a rabbit intermittently immunized with the antigen of recombinant PalB expressed from *A. oryzae* (Sigma–Aldrich) [52–54]. It was further purified by SDS-PAGE, and PalB used for immunization was obtained by polypepetide elution of the corresponding band in the polyacrylamide gel slice using an Electro-Eluter (Model 422; Bio-Rad). The secondary antibody was goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (HRP). PalB-related polypeptides were probed by a colorimetric method using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the substrate. The processed membrane was scanned.

Results

For the extracellular secretion of PalB from *E. coli*, the plasmid pEHLYAPalB containing the *palB-hlyA* gene fusion (encoding the protein fusion of PalB and the 23-kDa C-terminal of HlyA whose expression was regulated by the *lac* promoter) was constructed. The culture performance using JM109 harboring pEHLYAPalB and pVDL9.3 for shaker flask cultivation is summarized in Fig. 2. PalB bioactivity associated with the extracellular secretion of PalB-HlyA was visualized on tributyrin agar plates (Fig. 2a), indicating that HlyA can functionally mediate PalB secretion without affecting its proper folding. Two cultivation temperatures, i.e., 28 and 37°C, were used. While cell growth for the two cultures was similar (Fig. 2b), the 37°C culture had a higher specific PalB activity (128 vs. 83 U/L/OD_{600} in Fig. 2c). Therefore, all subsequent cultivations were performed at 37°C. Western blotting was also conducted to probe PalB-HlyA at approximately 58 kDa in both the extracellular and intracellular (soluble) fractions. While the PalB-HlyA band was detected in both fractions (Fig. 2d), PalB activity was measurable only in the extracellular fraction (Fig. 2a, c), but not the intracellular one (data not shown), implying that the secretion of PalB could assist proper protein folding to attain its bioactive form. Note that, for the later extracellular culture samples at 20 and 24 h, another PalB-related band with a molecular weight less than the expected size of 58 kDa was probed by PalB antibodies, implying that a possible proteolysis might have occurred on a specific cleavage site.

The effect of the inducer concentration on the secretion of PalB-HlyA was investigated (data not shown). The control experiment without IPTG induction had a specific PalB activity of 25 U/L/OD_{600} because of the expression leakage associated with the *lac* promoter. The PalB activity increased monotonically with the IPTG concentration up to 0.5 mM, at which the expression/secretion was optimal. Further increase in the inducer concentration had either no or an adverse effect on the culture performance. Nevertheless, the inducer concentration had no effect on cell growth, implying that the expression and secretion of PalB-HlyA posed a minimum physiological impact on producing cells. The doublet bands were still observed in extracellular culture samples taken at 20 and 24 h of growth.

Five *E. coli* hosts, i.e., BL21(DE3), DH5α, HB101, JM109, and MC4100, were used to observe any host effect, and the results are summarized in Fig. 3. While the specific PalB activity was the highest with the use of MC4100 or DH5α as the expression host (Fig. 3b), the latter suffered poor growth (Fig. 3a), which negatively impacted the overall culture performance. On the other hand, recombinant HB101 had the lowest specific PalB activity (Fig. 3b). Note that there was no detectable expression of PalB-HlyA in recombinant BL21(DE3) (data not shown), though it is often considered an effective host for recombinant protein production. The results suggest the importance of selecting a proper host strain for the extracellular production of PalB using the hemolysin secretion system. Interestingly, the doublet bands on the Western blot for the late culture samples of recombinant JM109 and DH5α were similar in pattern since the two bands had a comparable intensity (Fig. 3c). On the other hand, the lower band of the doublet for the late recombinant HB101 culture samples was minor, whereas the upper band of the doublet for recombinant MC4100 culture samples was minor (Fig. 3c). Note that the high PalB activities associated with the late recombinant MC4100 culture samples support the previous argument that both bands could represent a bioactive species.

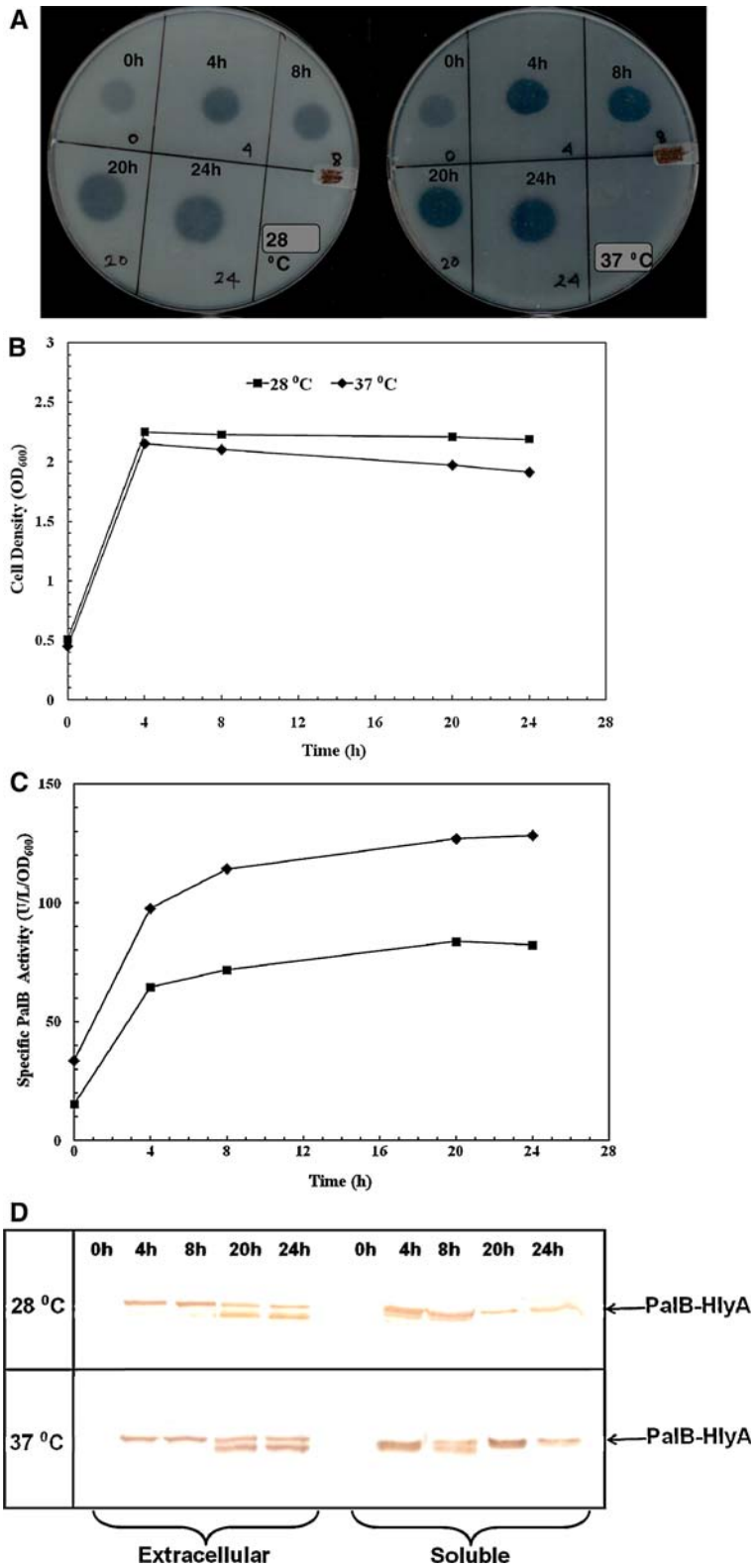

Fig. 2 Shaker flask cultivation of JM109 (pEHLYAPalB, pVDL9.3): The cultivation was conducted at 28 and 37°C. IPTG at 0.5 mM was used for induction. **a** Qualitative visualization of PalB activity using tributyrin agar plates. **b** Time profiles of cell density. **c** Time profiles of specific PalB activity. **d** Western blotting analysis of the extracellular and soluble intracellular fraction of culture samples

Extracellular secretion for functional expression of PalB was also explored in a bioreactor, and the cultivation results using JM109 (pEHLYAPalB, pVDL9.3) are summarized in Fig. 4. With the cultivation at 28°C, the specific PalB activity was increased by 50% at 130 U/L/OD_{600} as compared to the shaker flask culture in which the maximum PalB activity was 83 U/L/OD_{600}. On the other hand, only a slight improvement was observed in the secretion of

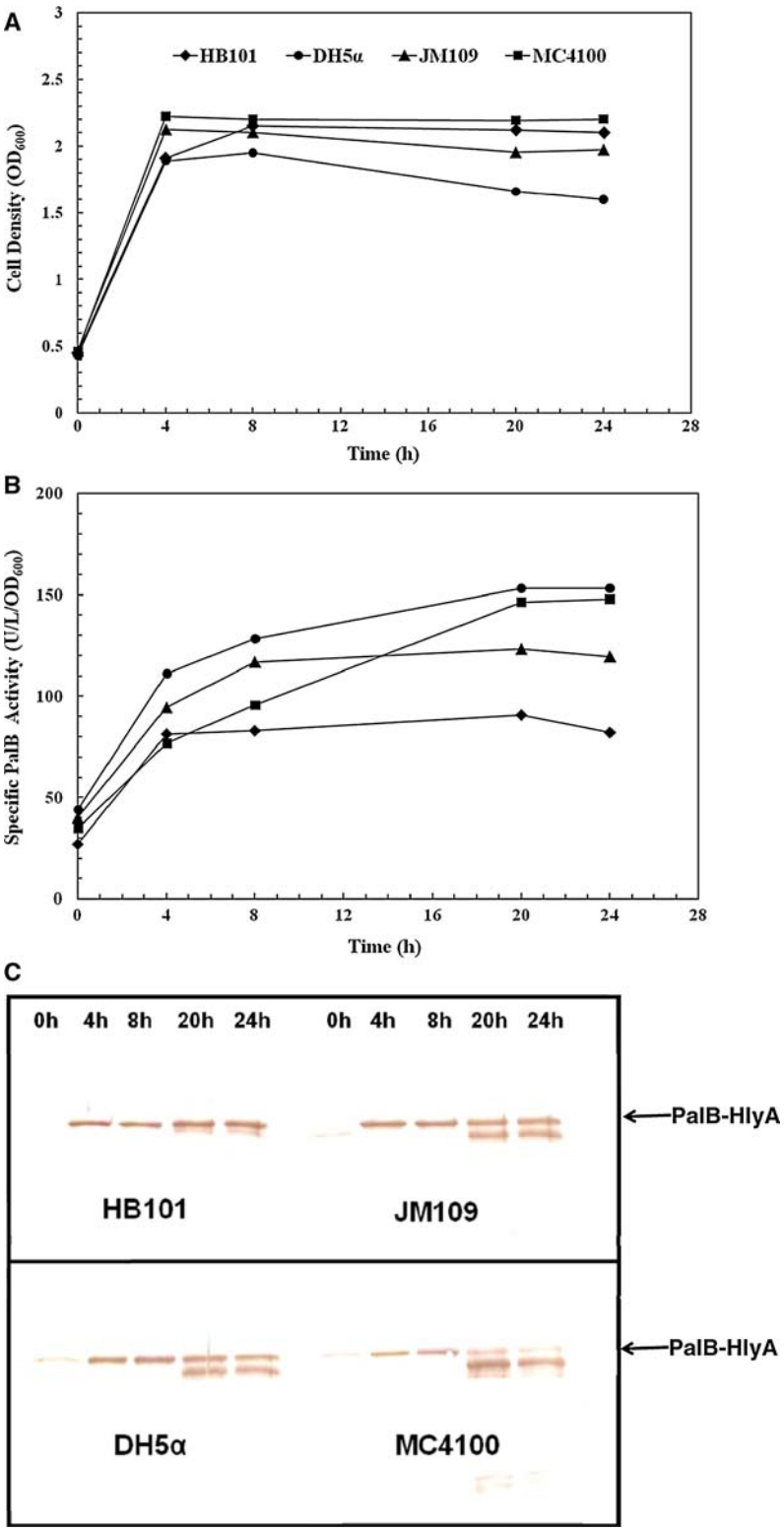

Fig. 3 Effect of the host cell on the culture performance for PalB-HlyA expression and secretion: Various *E. coli* hosts, including DH5α, HB101, JM109, and MC4100, harboring pEHLYAPalB and pVDL9.3 were used. The cultivation was conducted at 37°C with 0.5 mM IPTG. **a** Time profiles of cell density. **b** Time profiles of specific PalB activity. **c** Western blotting analysis of the extracellular fraction of culture samples

PalB-HlyA for the bioreactor culture at 37°C when compared to the shaker flask culture at the same temperature (i.e., 142 U/L/OD_{600} vs. 128 U/L/OD_{600}). While PalB-HlyA was effectively produced and secreted within 9 h of bioreactor cultivation at 37°C, the secreted protein appeared to be rather unstable, and the PalB activities declined after 9 h. The results imply that a lower cultivation temperature at 28°C might be favored for bioreactor cultivation, which appears to have a better ambience for faster secretion and production of PalB-HlyA.

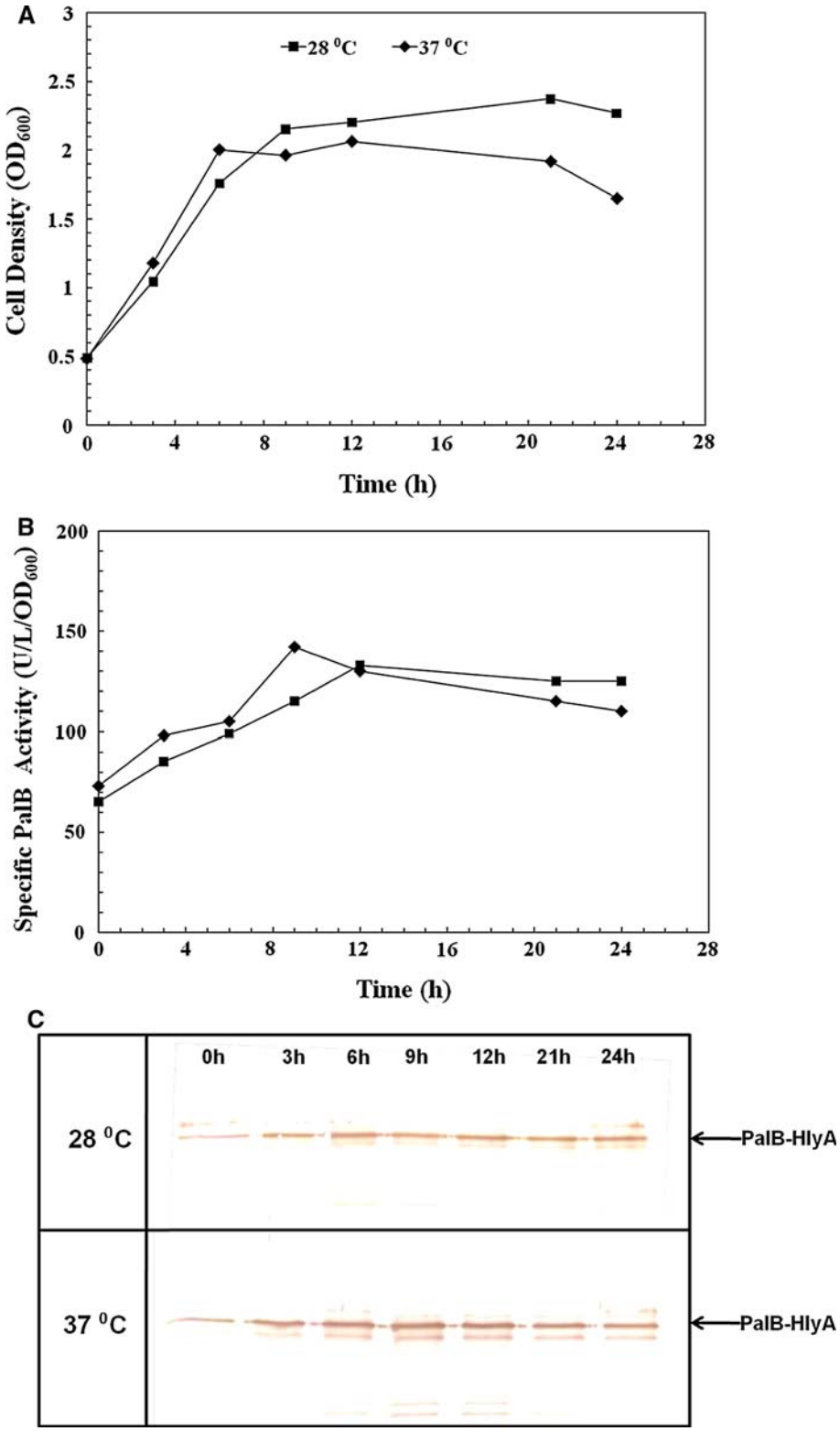

Fig. 4 Bioreactor cultivation of JM109 (pEHLYAPalB, pVDL9.3): The cultivation was conducted at 28 and 37°C. IPTG at 0.5 mM was used for induction. **a** Time profiles of cell density. **b** Time profiles of specific PalB activity. **c** Western blotting analysis of the extracellular fraction of culture samples

The modified flagellar secretion apparatus was also explored for the extracellular secretion of recombinant PalB using the plasmid p5′3′UTRPalB. The expression of PalB was under the regulation of the *fliC* promoter. Using *E. coli* MKS12, whose flagellin-related genes of *fliC* and *fliD* are deleted, as a host, the results of shaker flask cultivation are summarized in Fig. 5. PalB activity was absent in culture samples at growth times shorter than 24 h, consistent to the previous observation [27]. Hence, culture samples were collected during 24 and 48 h. Compared to

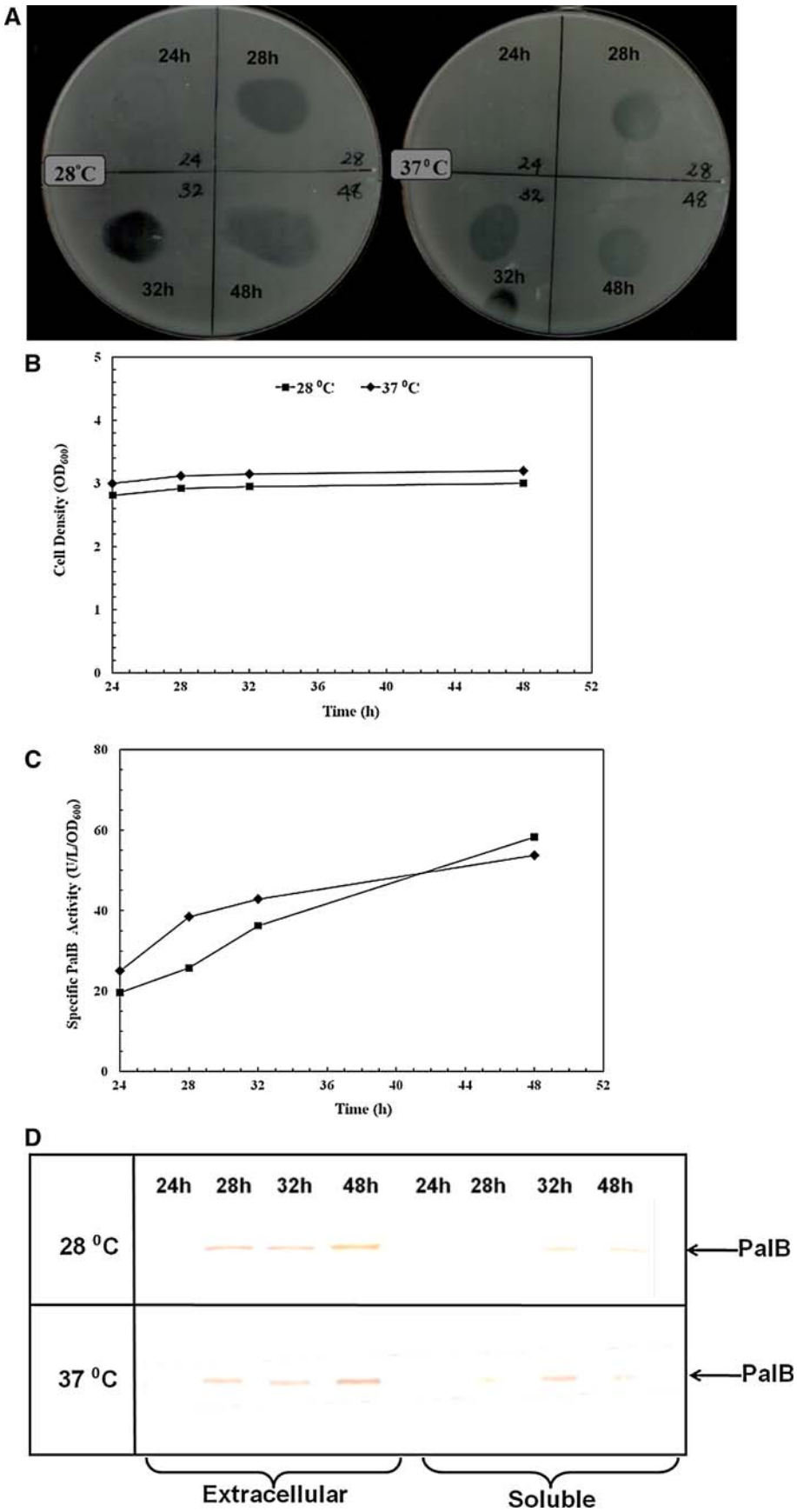

Fig. 5 Shaker flask cultivation of MKS12 (p5′3′UTRPalB): The cultivation was conducted at 28 and 37°C. **a** Qualitative visualization of PalB activity using tributyrin agar plates. **b** Time profiles of cell density. **c** Time profiles of specific PalB activity. **d** Western blotting analysis of the extracellular and soluble intracellular fraction of culture samples

the hemolysin system, the flagellar system appears to be less effective in terms of the secretion level, though the intact PalB rather than a PalB protein fusion was secreted. The cultivation was conducted at both 28 and 37°C with a rather similar culture performance in terms of cell growth (Fig. 5b) and the secretion level (Fig. 5c). Western blotting was conducted to probe the expressed PalB in both the extracellular and intracellular (soluble) fractions. While the PalB-related band at 35 kDa was observed in both fractions, PalB activity was present only in the extracellular fraction (Fig. 5c). The results suggest that extracellular secretion could assist proper folding for functional expression of PalB.

Culture performance for extracellular secretion of PalB was enhanced upon bioreactor cultivation, and the results are summarized in Fig. 6. Compared to the shaker flask cultivation at 37°C, the corresponding bioreactor culture had not only a higher cell density (5.8 vs. 3.2 OD_{600}), but also a higher specific PalB activity (70 vs. 54 U/L/OD_{600}), resulting in a significant enhancement in the volumetric PalB activity. There was also a slight improvement for bioreactor cultivation at 28°C due to a higher cell density compared to the shaker flask cultivation. Note that the secretion of PalB did not appear to pose any adverse effect on cell physiology based on the profile of cell growth.

Discussion

Though PalB has wide industrial applications, its functional expression in *E. coli* suffered various technical limitations. In our laboratory, various strategies were explored to express heterologous PalB in the cytoplasm, periplasm, and cell surface of *E. coli* with protein misfolding and intracellular proteolysis as the major expression issues [52–54]. Hence, extracellular secretion appears to be a plausible alternative for exploration. In this study, functional expression of heterologous PalB in *E. coli* was achieved by extracellular secretion via two Sec-independent pathways, i.e., the α-hemolysin (type I) and the modified flagellar (type III) secretion systems.

Since PalB has three disulfide bonds critical for developing the protein structure and biological activity, the absence of the PalB activity for the intracellular fraction of culture samples suggests that the intracellularly expressed polypeptides could be maintained in an unfolded or partially folded state presumably for preparing extracellular export. It was previously demonstrated that single chain Fv fragments secreted by the hemolysin transporter retained the antigen-binding activity, which is associated with the formation of correct disulfide bridges, upon the extracellular accumulation [13]. However, the *trxB* mutant of *E. coli* with an oxidizing cytoplasm had an inhibitory effect on the HlyA-dependant secretion of proteins with disulfide bonds [12]. These observations suggest that proper disulfide bonds formed upon either protein translocation through the export conduit or protein exposure to the oxidative environment outside the cell.

One of the advantages of the hemolysin transporter demonstrated in this study lies in its fast and efficient secretion of PalB-HlyA with no interference in the folding and activity of PalB from the HlyA moiety. The hemolysin transporter has been competitive for the secretion of small heterologous polypeptides, such as single Ig domains [7], single chain Fv [13], and Shiga-like toxin IIeB [47]. Apparently, the large size of PalB-HlyA with more than 500 amino acids did not affect its effective secretion, as demonstrated herein.

Though the hemolysin transporter is able to export the target protein extracellularly, it has at least two drawbacks. First, the secreted protein remains fused to HlyA and, depending on the application, an additional cleavage step might be required to obtain the native target protein. Second, coexpression of HlyB and HlyD secretion component proteins is required to mediate the secretion [1, 43]. The simultaneous overexpression of the target protein fusion (i.e., PalB-HlyA) and transport component proteins might lead to an extra physiological burden on producing cells, resulting in the relatively poor cell growth observed herein.

It is interesting to observe another protein species, with its size a bit smaller than the expected size of PalB-HlyA, in the extracellular fraction of culture samples after prolonged cultivation. Apparently, the secreted PalB-HlyA was subjected to extracellular proteolysis. In addition to intracellular proteases, cell envelope proteases can also mediate the proteolysis of recombinant proteins heterologously expressed in *E. coli*. For example, the outer membrane protease OmpT was shown to specifically degrade several recombinant proteins incubated with intact *E. coli* K-12 [18]. It was also applied for the proteolysis of secreted proteins fused with HlyA [20]. OmpT acts on dibasic residues [30] and is present in all the *E. coli* hosts used herein except BL21(DE3). The PalB-related band with a lower molecular weight could possibly represent the OmpT-degradation product of PalB-HlyA. Nevertheless, it seems that the extracellular proteolysis did not affect the PalB activity, particularly for the culture of recombinant MC4100 in which most of secreted PalB-HlyA was digested but without losing the PalB activity (Fig. 3c). Such proteolysis was almost negligible for the bioreactor cultivation at 28°C (Fig. 4c), implying that it could be associated with high cultivation temperature.

Type III secretion systems were originally identified when conducting studies on pathogenesis of bacteria whose invasion mechanism involves the injection of bacterial

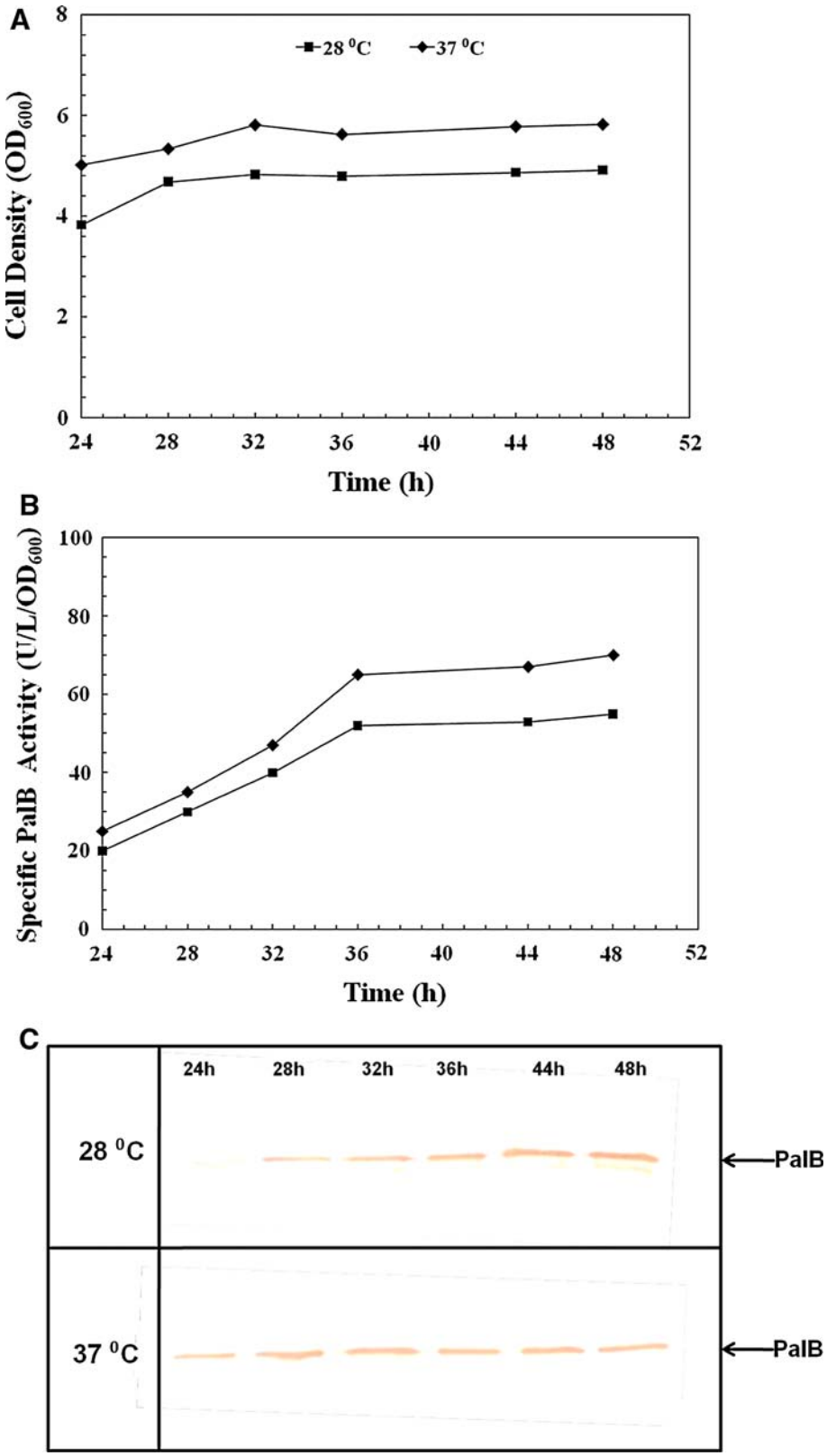

Fig. 6 Bioreactor cultivation of MKS12 (p5′3′UTRPalB): The cultivation was conducted at 28 and 37°C. **a** Time profiles of cell density. **b** Time profiles of specific PalB activity. **c** Western blotting analysis of the extracellular fraction of culture samples

proteins into host cells [14, 22]. The structural and regulatory components of the flagellum comprise the secretion apparatus, a type III secretion system that secretes the flagellum from the cytoplasm directly to the exterior of the cell [32]. Several proteins from evolutionarily distant sources, including Peb1 from *Campylobacter jejuni*,

D1-D3 repeats from *Staphylococcus aerus*, α-enolase from *Streptococcus pneumoniae*, and the eukaryotic green fluorescent protein, were successfully expressed in *E. coli* and extracellularly secreted using the flagellar secretion system. However, these studies also reported that there were polypeptides highly expressed intracellularly but hardly secreted, as well as those expressed at low levels but still secreted extracellularly.

While PalB secretion was achieved by the modified flagellar secretion system herein, the secretion rate was rather slow, and the expression/secretion level was relatively low. Majander et al. [27] reported that different target proteins have varying levels of protein yield that could be associated with the similarity of the target protein sequence to flagellin and in turn the efficiency of transcription and translation of the target protein gene. On the other hand, the secretion rate might be influenced by posttranslational folding since the flagellar subunits are secreted in an unfolded form [40] and fold only as they assemble [56]. However, such a secretion mechanism could be advantageous for functional expression of PalB, which requires the formation of three disulfide bonds for proper folding. Extracellular secretion of PalB apparently made disulfide bond formation more effective and proteolysis less susceptible, unlike our previous explorations with intracellular expression [52–54]. Given the slow secretion rate and relatively low PalB expression levels, the major advantage of the flagellar system is that the intact PalB, instead of PalB protein fusion as mediated by the hemolysin system, could be extracellularly secreted. This has a critical impact on biotechnological applications with respect to not only functional expression of PalB, but also simpler downstream purification and recovery.

It is also noteworthy, in both cases, that the extracellular secretion of PalB protein products posed a minimum physiological impact on producing cells since the cell growth profile was hardly affected by heterologous protein expression. Also, the expression/secretion performance appears to be enhanced by bioreactor cultivation as compared to the shaker flask cultivation, presumably due to the controlled environment conducive to producing cells. The extracellularly secreted PalB products were not subjected to major proteolysis and loss of bioactivity, which was previously observed upon intracellular expression [52]. While the secretion of PalB products was effective, both of the two secretion systems had a technical issue of low expression, which could be improved by using a strong promoter system.

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