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ORIGINAL ARTICLE

Novel synthetic signal peptides for the periplasmic secretion of green fluorescent protein in *Escherichia coli*

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Abstract New secretion vectors containing synthetic signal peptides were constructed to study the periplasmic translocation of green fluorescent protein (GFP) in *Escherichia coli*. These constructs encode synthetic signal peptides spA and spD fused to the amino terminal end of GFP, and expressed from T7/lac promoter in the BL21DE3 strain by induction with IPTG. The recombinant protein was detected in both the cytoplasmic and periplasmic fractions. Fluorescence analysis revealed that recombinant proteins with signal peptides were not fluorescent, indicating translocation to periplasmic space. In contrast, recombinant proteins without signal peptide were fluorescent. These results indicate that the expressed recombinant proteins were translocated into the periplasm. Therefore, the synthetic signal peptides derived from signal peptides of *Bacillus* sp. could efficiently secrete the heterologous proteins to the periplasmic space of *E. coli*.

Keywords Signal peptide · Periplasmic secretion · *Escherichia coli* · *Bacillus* sp · Green fluorescent protein (GFP)

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Introduction

Escherichia coli has been the workhorse for the production of various recombinant proteins due to simplicity of use, low cost, availability of various expression vectors, host strains, and extensive knowledge on its genetic characteristics (Baneyx 1999; Schmidt 2004).

Studies show that overexpressed proteins accumulate in *E. coli* cytoplasm and periplasm. Secretory production of recombinant proteins to the periplasm or extracellular medium is advantageous and eases downstream processing of the protein (Shokri et al. 2003). Secreted proteins have authentic N-terminal amino acid residues corresponding to their mature forms, subjected to less protease activity and allowing for simpler purification steps (Choi and Lee 2004).

Proteins destined for export are synthesized as precursors in the cytoplasmic compartment with a N-terminal extension known as signal peptides (SPs) (Heijne 1990). This transient peptide is cleaved off during or after translocation of export protein across or into the membrane by an integral membrane protease, signal peptidase (Heijne 1990; Suominen et al. 1995). The primary amino acid sequence of signal peptides is not conserved among various species (Heijne and Abrahmsen 1989), but they share some common characteristic features, which are conserved among different organisms. Most signal peptides are composed of three regions, the positively charged N-domain with 2–10 amino acids, the hydrophobic H-domain with 10 to 20 amino acids, and the C-domain that contains a cleavage site, which is most often an Ala-X-Ala box (Fekkes and Driessen 1999; Driessen and Nouwen 2008).

There is an increasing interest to identify new functional signal peptides for the development of an efficient secretory system. Interest in new signal peptides (SPs) also stems from the fact that they are critical in the secretion of target proteins as different signal peptides have different secretion efficiency. The secretion yield of industrially important proteins is often compromised due to the use of an incorrect SP sequence (Brockmeier et al. 2006). However, there is no universal signal peptide for the secretion of all target proteins and the selection of optimal SP sequences has to be done by trial and error. This is demonstrated by studies which show that the secretion of recombinant proteins in *E. coli* using various signal peptides including OmpA, LamB, OmpF and PelB did not always ensure efficient secretion (Choi et al. 2000).

Besides *E. coli*'s homologous SPs, other SPs especially from *Bacillus* sp. have been given considerable attention due to their secretory abilities. Furthermore, various extracellular enzymes from *Bacillus* sp. have been successfully expressed and secreted in *E. coli* (Manonmani and Kunhi 1999; Choi et al. 2000; Shahhoseini et al. 2003, Ong et al. 2008; Yamabhai et al. 2008; Low et al. 2010). The nature of *Bacillus* sp. as strong secretors makes its signal peptides desirable for secretion purposes in other hosts. Various studies have also shown that proteins from *Bacillus* sp. can be expressed in *E. coli* using their native signal peptides (Choi et al. 2000). These include expression of α-amylase (Manonmani and Kunhi 1999; Shahhoseini et al. 2003), CGTase (Yong et al. 1996; Kim et al. 1999; Ong et al. 2008), and mannanase (Zhang et al. 2006). These findings are interesting as they indicate that SPs from *Bacillus* sp. could function to target heterologous proteins in *E. coli*.

The functions of signal peptides are usually studied by fusing them to enzymes which can be screened using enzymatic assay. However, using GFP as a reporter would be more efficient due to its ability to produce fluorescence and its small size (27 kDa). To our knowledge, there have not been any studies on novel synthetic SPs in *E. coli*. Furthermore, the use of GFP has not been extended for the purpose of screening signal peptide functions in *E. coli*. This study aims to probe the ability of two synthetic signal peptides, spA and spD, derived from several *Bacillus* sp. in targeting heterologous proteins such as GFP to the periplasmic space of *E. coli*.

Materials and methods

Bacterial strains, plasmids and culture conditions

Escherichia coli TOP10 (Invitrogen, USA) was used as the host strain for cloning. *E. coli* strain BL21(DE3) (Invitrogen) was used as the expression host. Amplified PCR products were cloned into pCR®-Blunt II TOPO cloning vector and subsequently sub-cloned into pET-32b(+) (Novagen, USA) expression vector. Recombinant and non-recombinant *E. coli* cells were grown in LB broth at 37 °C with agitation at 250 rpm. Ampicillin was added at 100 μg/ml to maintain the recombinant plasmids.

Construction of signal peptides, spA and spD

Signal peptides of extracellular proteins α-amylase, β-mannanase and cyclodextrin glucanotransferase (CGTase) were analyzed by the ClustalW multiple alignment program to identify consensus amino acid residues which were made the backbone of new signal peptides. The designed signal peptides were then checked with the signal peptide prediction program SignalP 3.0 which is universally used for reliable prediction of signal peptides.

Molecular cloning of secretion cassette

Primers were designed for GFP and GFP fused with synthetic signal peptides. All primers are listed in supplementary table S1. The gene coding for green fluorescent protein (GFP) (714 bp) was amplified from the pET-32b(+) plasmid carrying the full length gene (Microbial Biotechnology Laboratory, Universiti Putra, Malaysia). Synthetic signal peptides spA and spD were amplified from synthesized oligonucleotides (i-DNA) using the primers listed in supplementary table S1. Thirty cycles of PCR were performed using *Pfu* DNA polymerase (Fermentas) to amplify both synthetic signal peptides and GFP genes. GFP secretion cassettes were constructed by ligating spA and spD with the GFP gene to form sp*GFP secretion cassettes. The secretion cassettes were cloned in pET-32b(+) expression vector, downstream of the T7 promoter, as shown in Fig. 1. The resulting plasmids, pGFP, pAGFP, and pDGFP, were transformed into *E. coli* BL21(DE3).

Expression and preparation of recombinant GFP from various fractions

Escherichia coli BL21(DE3) cells harboring constructs pGFP, pAGFP, and pDGFP (BL-pGFP, BL-pAGFP, and BL-pDGFP) were inoculated into 10 ml LB broth containing 100 μg/ml ampicillin and incubated at 37 °C for approximately 16 h with agitation at 250 rpm. One ml of the overnight culture was subcultured into 10 ml fresh LB broth supplemented with ampicillin and grown at 37 °C until the absorbance at OD_{600} reached 0.5. IPTG was added to a final concentration of 1 mM. The culture was incubated at 37 °C for 4 h with constant agitation at 250 rpm. The culture was then cooled prior to centrifugation at 8,000 *g* for 10 min at 4 °C to collect the supernatant and cells. The cells were used for the extraction of periplasmic proteins.

Osmotic shock method was applied for the extraction of periplasmic protein (Manonmani and Kunhi 1999). The cells were washed twice in half volume of culture with 10 mM

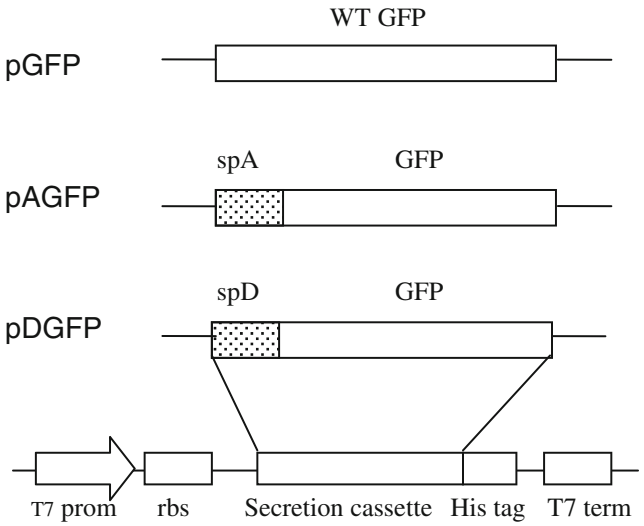

Fig. 1 ClustalW alignment of three signal peptides from *Bacillus* sp. for the identification of conserved residues for spA (**a**). ClustalW alignment of two *Bacillus* sp. signal peptides to construct spD (**b**). Residues in *blue* and *red* were made the backbone of the new signal peptides. *Blue* fully conserved residues, *red* strongly conserved residues

Tris–HCl (pH 8.0). The cells were resuspended in the same volume of 25 % sucrose. The suspension was shaken for 10 min at room temperature in 1 mM EDTA. After centrifuging at 8,000 *g* for 10 min, the cells are quickly and vigorously resuspended in the same volume of ice-cold water. This suspension was shaken for 10 min at 4 °C and centrifuged for 10 min at 9,000 *g*. The supernatant was carefully removed and labeled as periplasmic fraction. The pellet was kept for cytoplasmic protein extraction.

The cytoplasmic content from the remaining pellet was extracted by the glass bead method. The pellet was washed and resuspended in PBS prior to being lysed (0.1 g glass beads for 10 ml of culture). The cells were vortexed for 1 min for five times, each with a 1-min interval, on ice. The lysed cells were then centrifuged at 13,000 *g* for 10 min at 4 °C to collect the supernatant. The supernatant was labeled as cytoplasmic fraction.

Cell fractions were analysed by 12.5 % SDS-PAGE. Resolved proteins were transferred to PVDF membrane for immunoblotting using monoclonal anti-GFP rabbit primary antibody (Calbiochem) which was diluted 1:2,000 prior to incubation. Goat anti-rabbit IgG alkaline phosphatase (Calbiochem) diluted 1:2,000 was used as secondary antibody.

Expression of recombinant GFP corresponding to induction temperature and inducer concentration

Overnight cultures of *E. coli* BL21(DE3) cells harboring the constructs (pGFP, pAGFP, and pDGFP) were inoculated into 10 ml LB broth containing 100 μg/ml ampicillin at 37 °C in duplicate. Once the absorbance at OD_{600} reached 0.5, IPTG was added to a final concentration of 1 mM. The induced cultures were segregated and incubated at two different post-induction temperatures of 25C and 37 °C and protein expression and secretion were monitored by SDS-PAGE and western blotting as mentioned in "Molecular cloning of secretion cassette".

Fluorescence assay and microscopy

The translocation of recombinant GFP to periplasmic space was analyzed by fluorescence assay. The GFP clones, BL-pGFP, BL-pAGFP, and BL-pDGFP, and the negative control, BL21(DE3), were streaked on LB plates supplemented with ampicillin (100 μg/ml) and IPTG (1 mM/ml). The clones on the plates were then exposed to UV light to detect fluorescent clones. Cells harboring recombinant GFP clones were harvested following 4 h induction at 1 mM IPTG. The cells were washed and resuspended in PBS buffer, pH 7.4, and transferred to microscope slides. Expression of recombinant GFP was visualized using fluorescein isothiocyanate (FITC) filter in fluorescence microscopy and the phase contrast was also taken.

Results

Construction of synthetic signal peptides, spA and spD

The synthetic signal peptide, spA was constructed based on conserved residues from signal peptides α-amylase (*Bacillus licheniformis*), α-amylase (*Bacillus subtilis*), and CGTase (*B. licheniformis*) as shown in Fig. 2a. The conserved residues from signal peptides of β-mannanase (*B. subtilis*) and α-amylase (*B. licheniformis*) were used as the backbone for the second signal peptide, spD as shown in Fig. 2b. The identified fully conserved residues and strongly conserved residues were made the backbone of new synthetic signal peptides and the residues in black were chosen so as to fulfill the tripartite structure of signal peptides.

Construction and cloning of secretion cassettes

Synthetic signal peptides spA and spD were digested with *Bst*UI and ligated to the 5' end of *Bst*UI digested GFP gene to construct spAGFP and spDGFP secretion cassettes as shown in Fig. 1. Cassettes were further digested with *Bam*HI and *Hind*III to make compatible overhangs for ligation into the expression vector pET-32b(+). The new constructs were labeled as pAGFP and pDGFP, which harbored the spAGFP and spDGFP secretion cassettes, respectively. The 717-bp wild-type GFP gene was amplified with specific primers so that it resulted in deletion of the stop codon TAA at the 3' end of the gene. The GFP gene (714 bp) was digested with *Bam*HI and *Hind*III and ligated into pET-32b(+). This resulting

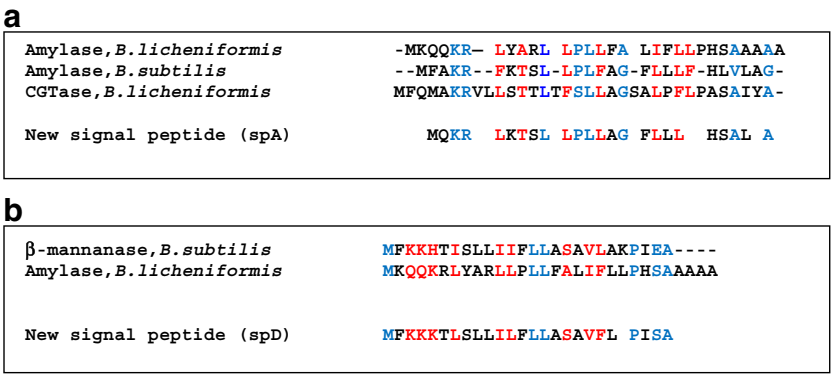

Fig. 2 Construction of sp*GFP secretion cassettes for recombinant GFP expression in *E. coli*. Construct pGFP contains wild-type GFP in pET-32b(+), construct pAGFP is a result of ligating spAGFP cassette into pET-32b(+), and pDGFP contains the spDGFP secretion cassette. The secretion cassettes were placed downstream of the T7 promoter and RBS site and upstream of the His tag

construct was labeled pGFP. The constructs were then transformed into *E. coli* BL21(DE3) and the positive transformants were selected for SDS-PAGE and western blotting analysis.

Expression of recombinant GFP in cytoplasmic and periplasmic compartment

In order to determine the ability of the new synthetic signal peptides to direct GFP to the periplasmic compartment of *E. coli*, SDS-PAGE and western blotting of cytoplasmic and periplasmic fractions were carried out. Expression of the recombinant GFP by the constructs pGFP, pAGFP, and pDGFP was analyzed after 4 h of induction with 1 mM IPTG at 37 °C.

After 4 h of induction, the target protein was detected in both cytoplasmic and periplasmic fractions in SDS-PAGE. The cytoplasmic fraction revealed the presence of GFP in both uninduced and induced samples at ~47 kDa for clones BL-pGFP, BL-pAGFP, and BL-pDGFP and a band at ~20 kDa for samples BL-pAGFP and BL-pDGFP (Fig. 3a). However, in the periplasmic fraction (Fig. 3b), distinct bands were observed for induced samples at ~20 kDa at lanes 5 and 7. Bands at 47 kDa were also present in the periplasmic fraction for induced samples at lanes 3, 5, and 7 in Fig. 3.

Effect of induction temperature and inducer concentration on periplasmic GFP secretion

To investigate the effects of post-induction temperature and inducer concentration on the expression of recombinant GFP into the periplasmic space, cells harboring the constructs were subjected to various inducer concentrations (0, 0.1, 0.5, and 1.0 mM) and two different culture temperatures (25 and 37 °C). SDS-PAGE analysis for the periplasmic fraction of clone BL-pAGFP shows bands of ~20 kDa at 0.1 mM and 1 mM inducer concentration for post-induction temperature of 37 °C. However, similar bands are not observed for clones with a post-induction temperature of 25 °C as shown in Fig. 4a. Clones BL-pDGFP (Fig. 4b) shows expected bands at ~20 kDa for the post-induction temperature of 37 °C, but this is not evident in the post-induction temperature of

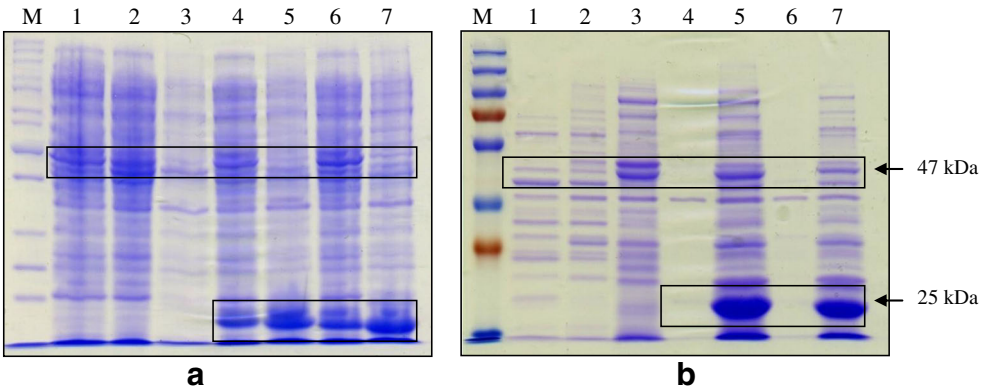

Fig. 3 SDS-PAGE analysis of recombinant GFP expression in cytoplasm (**a**) and periplasm (**b**). Expression and localization of GFP was observed in *E. coli* BL21(DE3) strain harboring pGFP, pAGFP, and pDGFP plasmids induced with 1 mM IPTG as described in "Materials and methods". *M* marker, *lanes 2*, *4*, and *6* are uninduced pGFP, pAGFP, and pDGFP samples, respectively. *Lanes 3*, *5*, and *7* are induced pGFP, pAGFP, and pDGFP samples, respectively. The *arrow* indicates recombinant GFP at two different sizes, the smaller fragment corresponding to the cleaved product

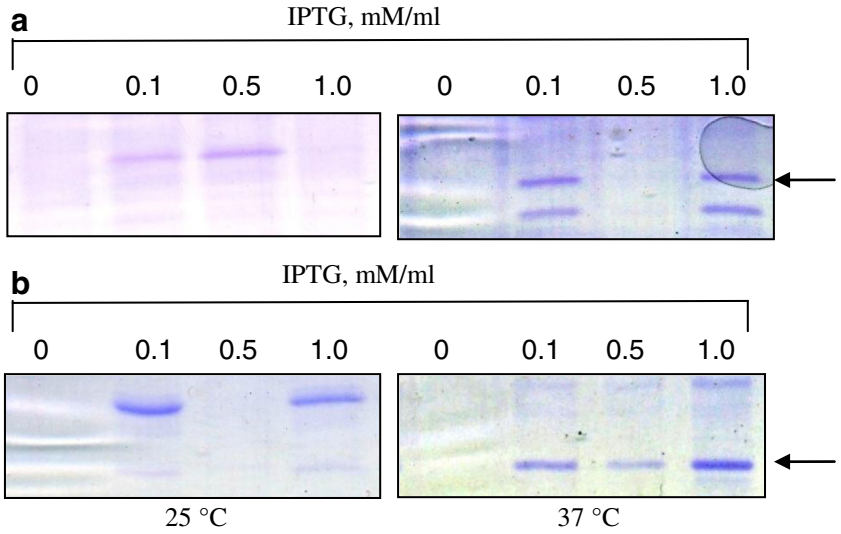

Fig. 4 SDS-PAGE analysis for recombinant GFP expression corresponding to various induction temperature and inducer concentration. Expression and localization of GFP was observed in *E. coli* BL21(DE3) strain harboring pAGFP and pDGFP plasmids induced with 0, 0.1, 0.5, and 1 mM IPTG as described in "Expression and preparation of recombinant GFP from various fractions". **a** Induction results for clone BL-pAGFP. **b** Induction results for clone BL-pDGFP. GFP expression was not observed at 25 °C but was observed for induction at 37 °C. The *arrow* indicates recombinant GFP at ~20 kDa

25 °C. Western blotting was carried out for clones induced with 1 mM IPTG at 25 and 37 °C for both BL-pAGFP and BL-pDGFP as this concentration gave better periplasmic expression as seen in SDS-PAGE. Results for induction at 37 °C revealed the presence of ~20-kDa bands for induced BL-pAGFP and BL-pDGFP as seen in lanes 2 and 4 of Fig. 5. This was not obtained for clones induced with similar IPTG concentration but at the lower temperature of 25 °C. Thus, western blotting analysis for the periplasmic fraction at post-induction 25 and 37 °C confirms SDS-PAGE results, as recombinant GFP is found to be secreted at 37 °C, but not at 25 °C.

Fluorescence assay and microscopy

Fluorescence analysis was carried out in this study to identify recombinant GFP activity in clones BL-pGFP, BL-pAGFP, and BL-pDGFP. The clones were streaked on LB Amp plates and were induced with IPTG. Induced clones were then exposed to UV light to detect fluorescent and non-fluorescent clones. Induced clone BL-pGFP, which lacked the N-terminal signal peptide, displayed fluorescence immediately as shown in Fig. S1 (2). However, clones BL-pAGFP and BL-pDGFP did not exhibit fluorescence as shown in Fig. S1 (3, 4).

The translocation of recombinant GFP to periplasmic space was also monitored by fluorescence microscopy. The fluorescence of clone BL-pGFP was compared with those of clones BL-pAGFP and BL-pDGFP. The recombinant clones were observed with ×1,000 magnification under FITC filter. Microscopy results showed that the BL21(DE3) harboring pGFP displaying intense fluorescence (Fig. 6, 1A). In contrast to this result, recombinant clones harboring pAGFP and pDGFP did not exhibit fluorescence as shown in Fig. 6, 2A and 2B.

Discussion

In this work, two new synthetic signal peptides were investigated for their ability to target the reporter protein GFP to the periplasm of *E. coli* BL21(DE3). The two signal peptides, spA and spD, were designed based on *Bacillus* sp. signal peptides to evaluate their ability in periplasmic translocation of target proteins in *E. coli*.

Bioinformatics study for the synthetic signal peptides was initially done using SignalP 3.0 software to identify their potential use as secretory signals. SignalP 3.0 is one of the most popular and widely used programs to analyze signal peptide probability and function. SignalP 3.0 identifies signal peptidase I cleavage sites and also classifies the proteins as secretory or non-secretory. This software uses neural network and hidden Markov models and can work on Gram-negative bacteria, Gram-positive bacteria, and eukaryotic sequences (Bendtsen et al. 2004). The SignalP 3.0 software utilizes discrimination scores (D-score) to distinguish between secretory and non-secretory proteins. Sequences with a D-score of above 0.5 are classified as signal peptides and those with a score of >0.7 have a higher probability to be signal peptides. Both spA and spD had D-scores of >0.6 which shows high potential to function as secretory signals. The signal peptidase I cleavage site for the SPs were also identified at A-X-A which is at the C-terminal end of the sequences. Thus, the SPs were further studied by fusing them to a reporter

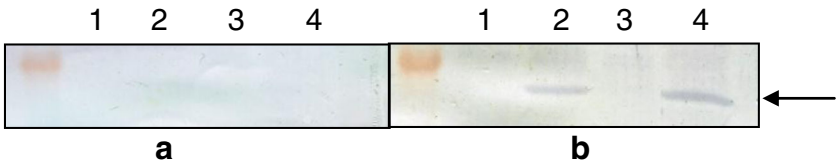

Fig. 5 Western blot analysis of recombinant GFP export to periplasm. Expression and localization of GFP was observed in *E. coli* BL21(DE3) strain harboring pAGFP and pDGFP with 1 mM IPTG as described in "Expression and preparation of recombinant GFP from various fractions". **a** Induction results of clone BL-pAGFP and BL-pDGFP at 25 °C. **b** Induction results of clone BL-pAGFP and BL-pDGFP at 37 °C. *Lanes 1* and *3* are uninduced BL-pAGFP and BL-pDGFP, respectively. *Lanes 2* and *4* are induced BL-pAGFP and BL-pDGFP clones, respectively. *Arrow* indicates recombinant GFP is expressed only in induced samples of BL-pAGFP and BL-pDGFP clones at 37 °C. The GFP expression was visualized using monoclonal anti-GFP rabbit primary antibody

protein. GFP was chosen as the reporter protein as it does not require any co-factors or substrates to exhibit fluoresence and can be easily viewed by fluorescence microscopy (Wu and Chung 2006). In addition, GFP have also been successfully used as a reporter for signal peptide selection (Drew et al. 2001), protein expression, and the monitoring of various cellular functions in prokaryotes (Dedieu et al. 2002; Wu and Chung 2006; Wilks and Slonczewski 2007).

SDS-PAGE and western blot analysis shows that the recombinant GFP was successfully translocated to the periplasm when fused to the new signal peptides, spA and spD. The cytoplasmically expressed GFP size is ~47 kDa. The expression vector used, pET-32b(+), has fusion tags upstream of the multiple cloning site which constitutes for the additional 20 kDa. This enables easy detection of periplasmically located recombinant GFP as the cleaved product would be ~27 kDa. Periplasmically expressed GFP was detected at ~25 kDa. SDS-PAGE and western blotting analysis also indicated that only induced samples were found to be targeted to the periplasmic space. The presence of some uncleaved recombinant GFP proteins (~47 kDa) in the periplasmic space could be due to overexpression and extracytoplasmic stress when induced

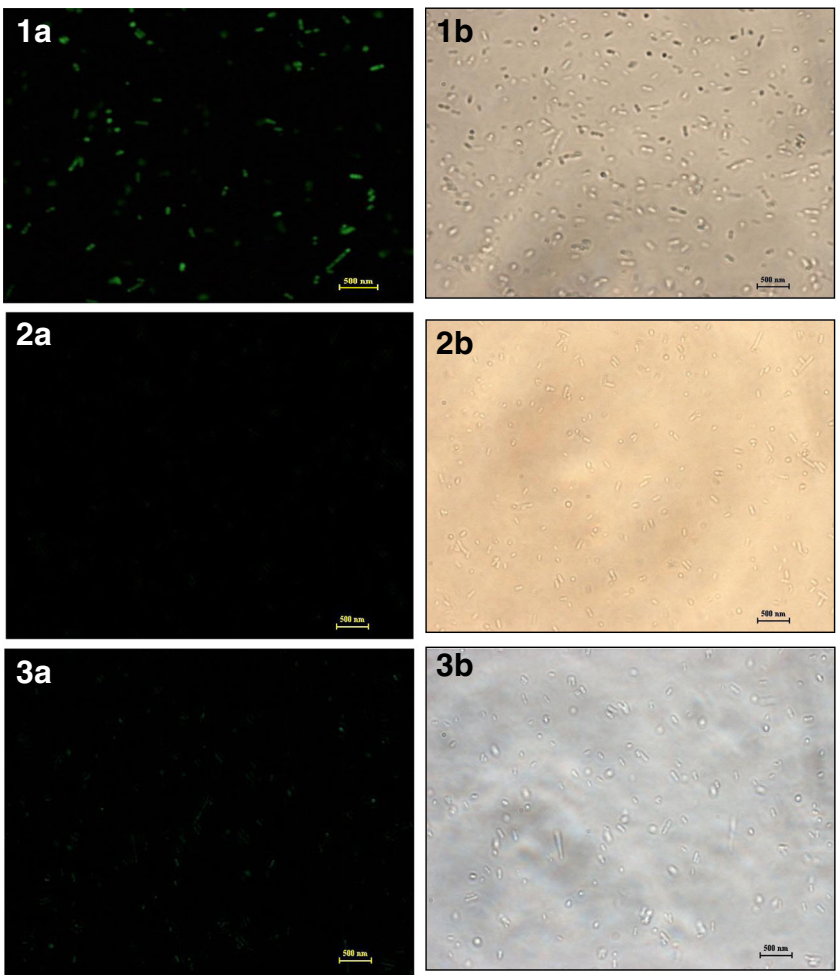

Fig. 6 Fluorescence microscopy image of cells expressing green fluorescent protein (GFP). Cells resuspended in 1× PBS were viewed immediately under FITC filter. *Panel 1A*, *2A*, and *3A* are fluorescent images. *Panels 1B*, *2B*, and *3B* are phase contrast images. *1A* and *1B* are *E. coli* BL21(DE3) harboring plasmid pGFP, *2A* and *2B* are *E. coli* BL21(DE3) harboring pAGFP, and *3A* and *3B* are *E. coli* BL21(DE3) harboring pDGFP

with IPTG, as it is not present in uninduced fractions in the periplasmic sample (Donovan et al. 1996).

We tried to further evaluate the effects of temperature and inducer concentration on the periplasmic secretion by the two synthetic signal peptides. Previous reports on GFP expression have found that lower temperature increases GFP expression and solubility (Drew et al. 2001; Omoya et al. 2004). In addition, 25 °C was chosen as induction temperature as it simulates the natural environment for GFP expression. Thus, *E. coli*'s optimum physiological temperature of 37 °C and a lower temperature of 25 °C were chosen for induction temperature analysis. SDS-PAGE and western blotting results showed bands at ~25 kDa at 37 °C but not at 25 °C (Figs. 4, 5). This is surprising as overall expression is much higher at 25 °C compared to 37 °C (not shown here). The lack of secretion at lower temperature might be linked to cell physiology rather than secretion machinery. The reduction in culture temperature has shown to reduce translocation as membrane goes from the liquid–crystalline bilayer to a more ordered gel (Shokri et al. 2003). These results indicate that protein secretion to the periplasmic space is temperature-dependent. Further analysis on inducer concentration reveals that higher periplasmic secretion was obtained for inducer concentrations 0.1 and 1.0 mM of IPTG. A higher concentration of IPTG has been shown to cause protein aggregation, and so a lower IPTG concentration is preferred for further downstream processing.

To further verify the secretory ability of synthetic signal peptides, fluorescence plate assay and microscopy was carried out to check GFP activity in recombinant cells. Fluorescence assay is a rapid screening method to identify GFP expression, as active GFP exhibits fluorescence under UV light exposure. Only clone BL-pGFP which expresses recombinant GFP cytoplasmically exhibited fluorescence under UV light. Clones BL-pAGFP and BL-pDGFP failed to fluoresce on the LB plate exposed to UV light. Similarly, fluorescent microscopy results also showed intense fluoresence for the recombinant clone BL-pGFP. However, clones BL- pAGFP and BL-pDGFP did not exhibit fluorescence when compared to the clone BL-pGFP. The lack of fluorescence could be explained by the presence of synthetic signal peptide in clones BL-pAGFP and pDGFP which prevented premature folding of the GFPs and makes them competent for subsequent translocation out of the cytoplasm. The translocated proteins are also unable to fluoresce in the periplasmic space. These results are similar to previous findings by Feilmeier et al. (2000), whereby GFPs ligated to maltose binding protein (MBP) signal peptide were found not to be fluorescent in the periplasm but produced fluorescence when the proteins were localised in the cytoplasm. Thus, they proposed that the GFP is unable to fold well and is inactive in the periplasm. This result was also substantiated by an investigation which showed GFP transported by the Tat pathway to be active in the periplasm as this pathway enables transport of fully folded proteins (Thomas et al. 2001). Taken together, our results show that recombinant GFPs are rendered inactive due to translocation to the periplasm and that this might explain the lack of fluorescence in BL-pAGFP and BL-pDGFP clones.

In conclusion, the newly designed synthetic signal peptides, spA and spD, were able to secrete recombinant GFP to the periplasmic space. Periplasmically secreted GFP was also found not to be fluorescent in the fluorescence assay. The induction of recombinant GFP expression also did not reduce or affect the growth rate and viability of GFP clones. In addition to the above, it has previously been reported that GFP is an efficient reporter system for identifying signal peptide function in *Lactobacillus reuteri* (Wu and Chung 2006). Thus, our results suggest that GFP vector design could provide a rapid and efficient tool to identify potential heterologous signal peptides for periplasmic secretion of recombinant proteins in *E.coli*, as it has not been attempted in the *E. coli* system before.

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