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Novel strategy for expression of authentic and bioactive human basic fibroblast growth factor in *Bacillus subtilis*

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Abstract

Inteins, also known as "protein introns," have been found to be present in many microbial species and widely employed for the expression and purification of recombinant proteins in *Escherichia coli*. However, interestingly, until now there has not been much information on the identification and application of inteins to protein expression in *Bacillus subtilis*. In this article, for the first time, despite the likelihood of absence of inteins in *B. subtilis*, this bacterium was shown to be able to facilitate auto-catalytic cleavages of fusions formed between inteins and recombinant proteins. Employing a construct expressing the intein, *Ssp* DnaB, (DnaB), which was fused at its N-terminus with the cellulose-binding domain (CellBD) of an endoglucanase encoded by the *cenA* gene of *Cellulomonas fimi*, the construct was demonstrated to be capable of mediating intracellular expression of basic fibroblast growth factor (bFGF), followed by auto-processing of the CellBD-DnaB-bFGF fusion to result in bFGF possessing the 146-residue authentic structure. The mentioned fusion was shown to result in a high yield of 84 mg l^{-1} of biologically active bFGF. Future work in improving the growth of *B. subtilis* may enable the use of this bacterium, working in cooperation with inteins, to result in a new platform for efficient expression of valuable proteins.

Keywords Human basic fibroblast growth factor · bFGF · Authentic structure · *Bacillus subtilis* · Inteins · *Ssp* DnaB · Fed-batch fermentation · Cellulose-binding domain

Introduction

Our group has been involved in the development of efficient bacterial platforms for the expression of recombinant proteins (Sivakesava et al. 1999; Fu et al. 2006; Wang et al. 2011; Kwong et al. 2013; Kwong and Wong 2013; Kwong et al. 2016a, b). Despite the success in exploiting *Escherichia coli* as a host for recombinant production of proteins, e.g., human basic fibroblast growth factor (bFGF; GenBank: AAV70487.1) (Kwong and Wong 2013; Kwong et al. 2016b) and human epidermal growth factor (EGF) (Sivakesava et al. 1999), which were previously shown to be difficult to be expressed efficiently as authentic products, *E. coli* suffers from the drawbacks of being an endotoxin producer and susceptible to high cell lethality due to plasmid curing (Lam et al. 1997; Sivakesava et al. 1999; Xu et al. 2000; Fu et al. 2005; Terpe 2006; Wang and Quinn 2010). As a result, these undesirable effects could make growth studies more complicated and difficult (Lam et al. 1997; Sivakesava et al. 1999; Xu et al. 2000; Fu et al. 2005).

Notwithstanding the aforementioned disadvantages of *E. coli*, our recent development of intein expression systems employing this bacterium as the host has revolutionized the expertise on recombinant production of proteins (Kwong and Wong 2013; Kwong et al. 2016a, b). The efficacies of the systems were studied using human bFGF as the protein model (Kwong and Wong 2013; Kwong et al. 2016b). Being a key member found in the basement membrane and sub-endothelial extracellular matrix, bFGF has been shown to possess different physiological functions and wound healing properties (Hoppenreijs et al. 1994; Vicario-Abejon et al. 1995; Unger et al. 2000; Akita et al. 2008, 2013; Okabe et al. 2013; Krejci et al. 2016). Due to its versatile applications, there has been a great demand for bFGF. Apparently, authentic bFGF (comprising 146 aa) is difficult to acquire, and therefore, only bFGF isoforms with different molecular sizes are commercially available. Despite the unauthentic structures of the isoforms, they may only be procured at an incredible high price (Gemini 2018; Peprotech 2018; Prospec 2018;

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Shenandoah 2018). Mediated by the self-cleavable *Saccharomyces cerevisiae* vascular membrane ATPase (VMA) intein (Cottingham et al. 2001; Ingham et al. 2005; Elleuche and Pöggeler 2010), we were successful in expressing the authentic mature bFGF product consisting of 146 aa in the cytoplasm of *E. coli* (Kwong and Wong 2013; Kwong et al. 2016b).

On the other hand, *Bacillus subtilis* appears to be an attractive alternative host for recombinant protein expression, although it is not as well characterized as *E. coli*. Being a Gram-positive bacterium, *B. subtilis* is free of endotoxins, and is thus perceived as a generally recognized as safe (GRAS) organism (Westers et al. 2004; Taguchi et al. 2015). In addition, recombinant *B. subtilis* strains have been shown to yield stable growth (Lam et al. 1998; Kwon et al. 2011), thus making optimization of product expression comparatively less complicated. Although *B. subtilis* has been employed to yield high levels of homologous proteins, e.g., α-amylase (Ikawa et al. 1998), it is yet a challenging task to achieve high-level expression of heterologous proteins in this bacterium (Li et al. 2004; Chen et al. 2015).

Over 600 putative intein genes have been discovered (Shah and Muir 2014) and many of them have been evaluated for use in mediating gene expression, essentially using *E. coli* as the host (Wu et al. 2011; Setrerrahmane et al. 2014). Previous findings showed that intein-mediated intracellular expression had been successful in resulting efficient production of proteins possessing authentic structures in *E. coli* (Kwong and Wong 2013; Kwong et al. 2016a, b), and that secretory expression was limited by the efficacy of the secretion signal employed as well as the channels available for transportation (Fu et al. 2005, 2006; Kwong and Wong 2013). Given the facts that *B. subtilis* has been employed successfully to express a wide collection of heterologous proteins, and that the bacterium does not produce endotoxins, it is tempting to exploit the intein-fusion approach for intracellular expression of heterologous proteins, which include in particular nutritionally or medically valuable proteins (Westers et al. 2004; Taguchi et al. 2015), in *B. subtilis*.

In this communication, we describe the development of a *B. subtilis* intracellular expression system, which employed the intein, *Ssp* DnaB, along with an endoglucanase cellulose-binding domain to facilitate successful production of bFGF as a soluble and precisely processed mature protein in the cytoplasm of *B. subtilis*. In addition, scale-up production of bFGF in fermentors showed that the recombinant culture maintained high levels of cell viability and plasmid stability, thus enabling a substantial improvement (~ 170%) of yield of bFGF. The findings support that the described *B. subtilis* intein-mediated expression approach may provide a practical solution for the production of toxin-free bFGF and, foreseeably, other medically valuable proteins.

Materials and methods

Bacterial strains and chemicals

E. coli strains ER2925 (NEB; Ipswich, MA, USA) and JM101 (Sivakesava et al. 1999) were used as intermediate hosts for recombinant DNA manipulations. *B. subtilis* strain 1A751 was described previously (Kwong et al. 2013). The Phusion PCR Kit, restriction, and modifying enzymes were purchased from NEB (Ipswich, MA, USA). All oligos were purchased from Invitrogen (Carlsbad, CA, USA). Chemicals used in this study were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise specified. Antibodies against bFGF were raised in-house in rabbits.

Engineering of constructs expressing fusions comprising intein *Ssp* DnaB and bFGF

The engineering of construct pM2-DnaB-bFGF was achieved using many steps and rounds of overlap extension PCR summarized as follows. Firstly, with oligos P1 and P2 (Table 1) as primers and a derivative of plasmid pM2VegCenA (Lam et al. 1998), pFC, which was extended by PCR to regain the 5′-terminal 1–45 codons (deleted in pM2VegCenA) of the full-length *cenA* gene (Wong et al. 1986), as the template, a fragment comprising the *vegC* promoter, *lac* operator, and ribosomal-binding site (RBS) of *B. subtilis* (Product 1-1) was attained. Secondly, using oligos P3 and P4 (Table 1) as primers and plasmid pTWIN1 (NEB; Ipswich, MA, USA) as the template, a sequence with the intein gene for *Ssp* DnaB and nucleotides overlapping those at (i) the 3′ end of Product 1-1 and (ii) the 5′ end of the coding sequence for bFGF (Product 1-2) was obtained. Thirdly, with oligos P5 and P6 (Table 1) as primers and pWK3R (Kwong and Wong 2013) as the template, a fragment containing the coding sequence for bFGF fused with a partial sequence of *Ssp* DnaB (Product 1-3) was generated. All Products were purified and subjected to a second round of overlap extension PCR. To obtain a precise fusion (Product 2-1) between the sequences coding for *Ssp* DnaB and bFGF, oligos P3 and P6 (Table 1) were used as primers, while Product 1-2 and Product 1-3 were employed as templates. Similarly, using oligos P1 and P6 (Table 1) as primers, along with Products 1-1 and 2-1 as templates, a 1.02-kb *Eco*RI-*Xba*I fragment (Product 3-1) comprising the following components: *vegC* promoter, *lac* operator, RBS of *B. subtilis*, coding sequences for intein *Ssp* DnaB, and bFGF, was obtained. Last of all, Product 3-1 was digested with *Eco*RI and *Xba*I, followed by ligation with a *B. subtilis*/*E. coli* shuttle vector, pM2-Veg (Lam et al. 1998), which had been digested with the same two restriction enzymes to result in construct pM2-DnaB-bFGF.

Table 1 Primers employed in this study

Primer	Orientation	Sequence(5′ to 3′)*
P1: *Eco*R1 VegC F	Forward	AAAGAATTCTAATTTAAATTTTATTTG
P2: VegC DnaB R	Reverse	CATTTTTTATCACCTC
P3: VegC DnaB F	Forward	GAGGTGATAAAAAATGTGTATCTCTGGCGATAG
P4: DnaB-bFGF R	Reverse	CTCTGGCAAGGCTGGGTTGTGTACAATGAT
P5: DnaB-bFGF F	Forward	ATCATTGTACACAACCCAGCCTTGCCAGAG
P6: *Xba*I-bFGF R	Reverse	AATTTCTAGATCATTATTAGCTCTTAGCAG ACATT
P7: VegC CellBD R	Reverse	GCAGCCGGGAGCCATTTTTATCACCTCCTT
P8: VegC CellBD F	Forward	AAGGAGGTGATAAAAATGGCTCCCGGCTGC
P9: CellBD-DnaB R	Reverse	ATCGCCAGAGATACAGGTGCCCGTGCAGGT
P10: CellBD-DnaB F	Forward	ACCTGCACGGGCACCTGTATCTCTGGCGAT

*Restriction sites used in the cloning experiments are underlined

To construct expression construct pM2-CellBD-DnaB-bFGF, overlap extension PCR was performed, using oligos P1, P6, and P7–P10 (Table 1) as primers, and plasmids pFC and pM2-DnaB-bFGF (both described above) as templates. In doing so, the DNA sequence encoding the cellulose-binding domain (CellBD) of an endoglucanase encoded by the *cenA* gene of *C. fimi* {GenBank: M15823.1; Wong et al. 1986)} was cloned upstream of the DnaB-bFGF DNA fusion to obtain pM2-CellBD-DnaB-bFGF.

Protein expression, purification, and analysis of the bFGF product

Shake flask cultivation

A modified version of the MBL medium (MMBL) for growth of *B. subtilis* transformants in shake flasks was described previously (Sivakesava et al. 1999; Kwong and Wong 2013). To prepare seed cultures of the two transformants containing constructs pM2-DnaB-bFGF and pM2-CellBD-DnaB-bFGF, a fresh colony of each transformant was grown in 100 ml of MMBL supplemented with 20 μg ml^{-1} of kanamycin. The culture was then grown at 250 rpm and 37 °C until the A_{600} reading reached 8.0, followed by an addition of a final concentration of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the growing cells. Culture samples were then collected at 2-h intervals for the analysis of bFGF expression.

Preparation of cell lysates

The cell pellets were each re-suspended in 120 μl of Tris-HCl buffer (50 mM, pH 8.0), followed by an addition of 83 μl of EDTA solution (0.25 M, pH 8.0) and incubation on ice for 5 min. The cells were then treated with 120 μl of lysozyme solution (10 mg ml^{-1}) at 37 °C for 20 min. To enhance cell lysis, 83 μl of solution X (10 mM EDTA, 10% Triton X-100, and 50 mM Tris-HCl, pH 8.0) was added, followed by gentle inversion of the tubes for 50 times. After centrifugation at 13,000 rpm for 10 min, lysate samples were collected and analyzed by Western blotting for bFGF expression as described previously (Kwong and Wong 2013). The images were quantified by densitometry using the ImageJ software (National Institutes of Health, USA).

Fed-batch fermentation

To prepare the seed culture, *B. subtilis* [pM2-CellBD-DnaB-bFGF] cells were grown in MMBL (Kwong et al. 2016b) supplemented with 20 μg ml^{-1} of kanamycin at 250 rpm and 37 °C until the A_{600} reading reached 1.0. Afterwards, 15 ml of the seed culture was transferred to a 500-ml Erlenmeyer flask containing 135 ml of fresh MMBL supplemented with 20 μg ml^{-1} of kanamycin, and the culture was further grown at 250 rpm and 37 °C until the A_{600} reading was 1.0. The entire 150 ml of seed was added into a 2-l fermentor containing 1.35 l of MMBL supplemented with 20 μg ml^{-1} of kanamycin, with an addition of 1 M NaOH to maintain the pH value of the culture at ~6.8. When the glucose was depleted and the pH began to increase, the culture was fed at 30-min intervals with 2 ml of 50% (*w*/*v*) glucose solution. The speed of the impeller was set at 600 rpm to help improve pO_2 in the culture. When the pO_2 value dropped to a level of about 30%, a mixture of compressed air and pure oxygen adjusted at a ratio of 50/50 was used to improve pO_2 in the fermentor. The feeding was continued until the A_{600} reading reached 20. When the pH of the culture turned to 6.8, it was induced with a final concentration of 0.5 mM IPTG. Culture samples were then collected at 2-h intervals for the analysis of bFGF expression.

Purification and amino acid sequencing of bFGF

Heparin-agarose chromatography was employed to purify bFGF present in cell lysates as described previously (Kwong

et al. 2013; Sivakumar et al. 2018). Purified bFGF was visualized on a SDS-PAGE gel stained with Coomassie blue. A band containing bFGF retrieved from the gel was analyzed by liquid chromatography tandem mass spectrometry as described previously (Kwong et al. 2013).

Bioassays for bFGF

Interaction between bFGF and its receptors will activate intracellular signal transduction pathways. Stimulation and induction of tyrosyl-phosphorylation of fibroblast growth factor receptor substrate 2α (FRS2α) in C2C12 myoblasts (from American Type Culture Collection, Manassas, VA) by bFGF was performed as described previously (Kwong et al. 2013). C2C12 cells treated with the same quantity of bFGF derived from *E. coli* [pWK311ROmpAd] and *B. subtilis* [pM2-CellBD-DnaB-bFGF] transformants, employing phosphate buffer as the negative control, were harvested and lysed. FRS2α was immunoprecipitated and analyzed by Western blotting with the help of anti-FRS2α (internal control) and anti-phosphotyrosine antibodies (Kwong et al. 2013). On the other hand, based on its mitogenic effect on the proliferation of BALB/c 3T3 fibroblast cells rated using the MTT assay as described previously (Kwong and Wong 2013), the potency of bFGF could also be determined.

Results

Rationale for engineering the expression plasmids concerned

Since not much is known regarding how inteins operate in *B. subtilis*, it was reckoned that our experience gained in intein-mediated expression of heterologous proteins in *E. coli* might shed light on the engineering of self-cleavable fusions formed between inteins and target proteins in *B. subtilis*. From previous studies, it was noted that the presence of both the N- and C-exteins embracing an intein could somehow enhance the success in achieving expression of a soluble fusion product formed between an intein and a foreign protein, irrespective of whether the target protein was expressed at the N- or C-terminus of the intein (Kwong and Wong 2013; Kwong et al. 2016a, b). To facilitate the recovery of the resulting intermediate fusion, which was hopefully retrievable using a facile protocol, it was decided that an 11-kDa CellBD of an endoglucanase (Eng) encoded by the *C. fimi cenA* gene (Wong et al. 1986) was exploited. Since CellBD was able to bind to cellulose (Wong et al. 1986; Din et al. 1994) and it was located at the N-terminal portion of Eng, it was expected that it might be expressed and act as an N-extein to provide the required anchorage for retrieving the intermediate fusion, which might exist as an uncleaved insoluble product. To study whether bFGF expression might be facilitated by the target intein, DnaB, two constructs, designated pM2-DnaB-bFGF (6.5 kb), which lacked the DNA coding sequence for CellBD (Fig. 1), and pM2-CellBD-DnaB-bFGF (6.8 kb), which harbored the DNA sequence concerned (Fig. 1), were engineered.

Expression of bFGF in *B. subtilis*

Results of time course experiments (Fig. 2) showed that construct pM2-DnaB-bFGF (Fig. 1) expressed only low levels of bFGF derived from cleavages of precursor/intermediate fusions (P/I). Despite the weak expression, the auto-cleavable activities appeared to be efficient, thus leaving almost undetectable trace of P/I (data not shown). It was then recollected that the presence of "an N-extein" such as EGF might enable P/I to achieve extendable and cleavable structures (Kwong and Wong 2013; Kwong et al. 2016a, b), as it was shown previously that EGF facilitated EGF-VMA-bFGF to achieve efficient expression and subsequent auto-processing to obtain authentic bFGF as the final product in *E. coli* (Kwong and Wong 2013; Kwong et al. 2016b). However, the same

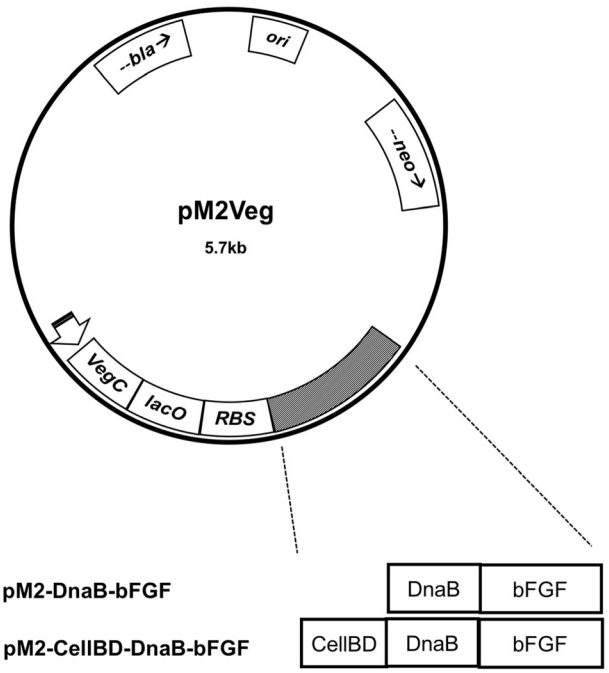

Fig. 1 Schematic representation of DNA constructs expressing the intein, DnaB, fused with different protein molecules. The top diagram shows vector pM2Veg (5.7 kb) with different DNA coding sequences inserted as shown below to form plasmids: (a) pM2-DnaB-bFGF (6.5 kb) and (b) pM2-CellBD-DnaB-bFGF (6.8 kb), which were expected to result in the expression of P/I: DnaB-bFGF and CellBD-DnaB-bFGF, respectively. Symbols for genetic elements shown in pM2Veg and its derivatives are the following: *ori* = replication origin of *B. subtilis*; *neo* = structural gene conferring resistance to neomycin; *bFGF* = bFGF gene; *VegC* = *vegC* promoter; *lacO* = *lac* operator. Arrows indicate directions of gene expression

approach of employing EGF to result in the same P/I: EGF-VMA-bFGF, did not result in successful expression of bFGF in *B. subtilis* (data not shown). It was then speculated that another pair of N-extein-intein fusion might yield better results. The 11-kDa CellBD, which was twice as big as EGF and was applied previously as a fusion tag (Greenwood et al. 1994), was considered for use to replace EGF. With the employment of CellBD, protein expression was expected to yield CellBD-DnaB-bFGF (Fig. 1(b) as P/I. Encouragingly, the replacement of CellBD-DnaB for EGF-VMA resulted positively in bFGF expression. In this connection, much higher levels of bFGF were detected in culture samples of *B. subtilis* [pM2-CellBD-DnaB-bFGF] transformant (Fig. 2b) than those of its counterpart harboring construct pM2-DnaB-bFGF, which lacked the coding sequence for CellBD (Fig. 2a).

Time course expression of bFGF

Results from initial shake flask cultivation supported that construct pM2-CellBD-DnaB-bFGF was able to yield higher levels of bFGF when expression was achieved under induction (Fig. 2b). Time course experiments were then undertaken to obtain a more complete picture of bFGF production resulting from induced expression of pM2-CellBD-DnaB-bFGF in *B. subtilis*. Two pieces of useful information were obtained from the study. First, IPTG induction worked well to provide not only increasing but also a fruitful expression of up to 31 mg l^{-1} of bFGF (Fig. 3). Second, this approach of expression resulted in also improved levels of bFGF-specific activity (Fig. 3b). Apparently, both the stable cell growth of *B. subtilis* [pM2-CellBD-DnaB-bFGF] and the lack of plasmid curing (Fig. 3b) contributed much to maintain the bFGF-specific activity at a high level.

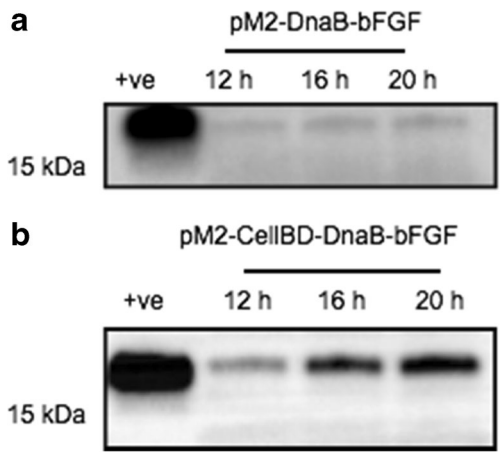

Fig. 2 Western blot analysis of recombinant bFGF expressed in lysate samples of *B. subtilis* cultures. Culture samples of **a** *B. subtilis* [pM2-DnaB-bFGF] and **b** *B. subtilis* [pM2-CellBD-DnaB-bFGF] transformants grown under IPTG induction were collected from different time intervals and analyzed. Lane +ve: bFGF standard (0.5 μg); lanes 12 h, 16 h, and 20 h: samples collected from cultures induced for 12, 16, and 20 h, respectively; each lane was loaded with 10 μl of cell culture

Fermentative production of bFGF

It was expected that improved levels of dissolved oxygen supplied to the culture medium of *B. subtilis*, which is a strictly aerobic bacterium, would lead to enhanced cell growth, and hence higher yields of the target recombinant product. In view that MMBL medium and fed-batch fermentation conditions worked well for intein-mediated expression of recombinant proteins in *E. coli* (Kwong et al. 2016b), the efficacy of scale-up expression of bFGF mediated by construct pM2-CellBD-DnaB-bFGF in *B. subtilis* cultivated in 2 l fermentors using the same methodology was investigated.

The results of the fermentation study showed remarkable improvements in both the levels of bFGF expression and cell density of the *B. subtilis* [pM2-CellBD-DnaB-bFGF] culture. The maximum yield of bFGF increased from 31 mg l^{-1}, expressed using shake flask cultivation (Fig. 3), to 84 mg l^{-1}, obtained from fermentative production (Fig. 4). Moreover, there was a fivefold increase in the final cell density of the culture in scaling up from shake flask cultivation (Fig. 3)

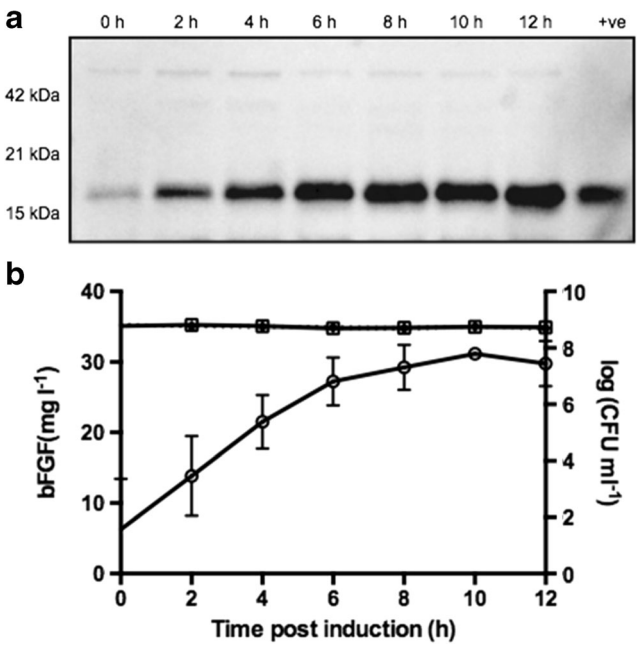

Fig. 3 Time course study of bFGF expression in *Bacillus subtilis* [pM2-CellBD-DnaB-bFGF] cells grown in shake flask. Samples were collected from the culture grown under IPTG induction at different time intervals. **a** Western blotting of recombinant bFGF present in lysate samples. All the wells were loaded with a sample size equivalent to 10 μl of the cell culture. **b** Quantitative analysis of bFGF and cell viabilities. Levels of bFGF detected in the cell lysates (—o—) are presented. Viabilities of plasmid-free and plasmid-containing cells were determined on plain agar plates (—■—) and plates supplemented with kanamycin (···◆···), respectively. CFU refers to colony-forming units. Growth experiment of the transformant was repeated three times and standard error bars are shown

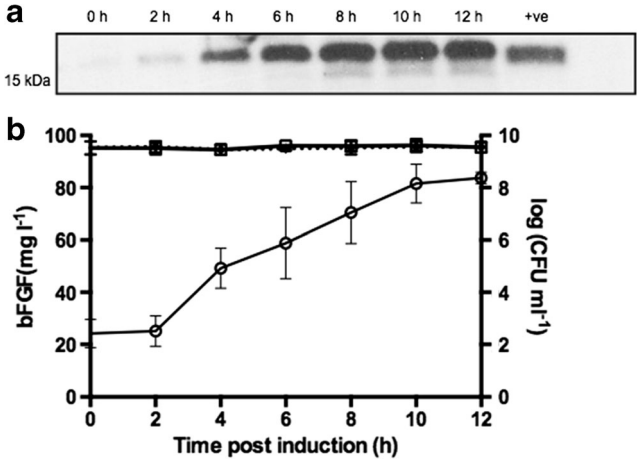

Fig. 4 Time course study of bFGF expression in *Bacillus subtilis* [pM2-CellBD-DnaB-bFGF] cells cultivated in a 2-l fermentor. **a** Western blot analysis of recombinant bFGF present in lysate samples. All the wells were loaded with a sample size equivalent to 5 μl of the cell culture. **b** Quantitative analysis of bFGF and cell viabilities. Samples were collected from the culture grown under IPTG induction at different time points. Levels of bFGF detected in the cell lysates (—o—) are presented. Viabilities of plasmid-free and plasmid-containing cells were determined on plain agar plates (—■—) and plates supplemented with kanamycin (···◆···), respectively. CFU refers to colony-forming units. Growth experiment of the transformant was repeated three times and standard error bars are shown

to small-scale fermentation (Fig. 4). Presumably due to the use of improved oxygen supply, refined mode of feeding, and pH condition for growth ("Materials and methods"), the *B. subtilis* [pM2-CellBD-DnaB-bFGF] culture was grown to achieve a high cell density, reaching a value of nearly 10^{10} cells ml^{-1}, even when bFGF expression was carried out under IPTG induction (Fig. 4). This high density, which was five times more than that attained from induced expression in shake flasks, resulted in a satisfactory yield, 84 mg l^{-1}, of bFGF despite the absence of accelerated cell growth throughout the entire time course study. Although the cells employed for induced expression had apparently entered the stationary phase, they remained intact even when bFGF was actively expressed. More attractively, the pM2-CellBD-DnaB-bFGF construct was stably maintained in its host cells (Fig. 4). This observation was strikingly different from that displayed by *E. coli* transformants experiencing fermentative expression of recombinant proteins, during which dramatic plasmid loss was detected (Sivakesava et al. 1999; Kwong and Wong 2013; Kwong et al. 2016b).

The primary structure of bFGF expressed in *B. subtilis*

Western blot analysis (Fig. 4) revealed that recombinant bFGF retrieved from the lysate of *B. subtilis* [pM2-CellBD-DnaB-bFGF] cells possessed the same molecular size as that of the 146-residue bFGF standard (Kwong et al. 2016b). However, it was not yet certain whether the bFGF product resulting from auto-cleavages of P/I consisted of the expected 146 aa (Kwong et al. 2013; Kwong and Wong 2013; Kwong et al. 2016b). A purified bFGF sample was then retrieved from the last time point of a fermentation culture and subjected to analysis by mass spectrometry (Kwong et al. 2013). The results of the analysis, for the first time, demonstrated that *B. subtilis* was able to facilitate in vivo auto-cleavages of an intein fusion P/I: CellBD-DnaB-bFGF, to yield the desired product, bFGF, possessing the 146 aa authentic structure (Table 2).

Mitogenicity of recombinant bFGF

Reminiscent of the mitogenic effect demonstrated by authentic bFGF recovered from *E. coli* transformants (Kwong and Wong 2013; Kwong et al. 2016b), recombinant bFGF expressed by the intein DNA construct, pM2-CellBD-DnaB-bFGF, in *B. subtilis* was also shown to be biologically active (Fig. 5). Comparison between bFGF samples obtained from recombinant *E. coli* and *B. subtilis* cells revealed that they showed comparable potency (Fig. 5).

Discussion

Since the first intein was discovered in the late 1980s (Hirata et al. 1990), the cloning and characterization of intein genes has not only enabled us to better understand the molecular and biochemical functions of inteins, but also facilitated our employment of them to mediate expression of recombinant proteins with structures possessing the expected aa compositions (Elleuche and Pöggeler 2010; Kwong and Wong 2013; Kwong et al. 2016a, b). *E. coli*, being the most common host for heterologous gene expression, has also been the most commonly employed organism for recombinant protein expression mediated by inteins (Kwong and Wong 2013; Kwong et al. 2016a, b). This relatively

Table 2 Analysis of purified bFGF by liquid chromatography tandem mass spectrometry

Constructs	Peptide[a]	Mr(Calc)[b]	Mr(Expt)[c]	Ion score
CellBD-DnaB-bFGF	-PALPEDGGSGAFPPGHFKD	1779.858	1780.0282	40
	KAILFLPMSAKS-	1105.6205	1105.9454	59

[a] Subsequent to partial trypsin digestion of bFGF, the N-terminal and C-terminal sequences were identified by the Mascot search engine

[b] Theoretical mass-to-charge ratio of the peptide

[c] Experimental mass-to-charge ratio of the peptide

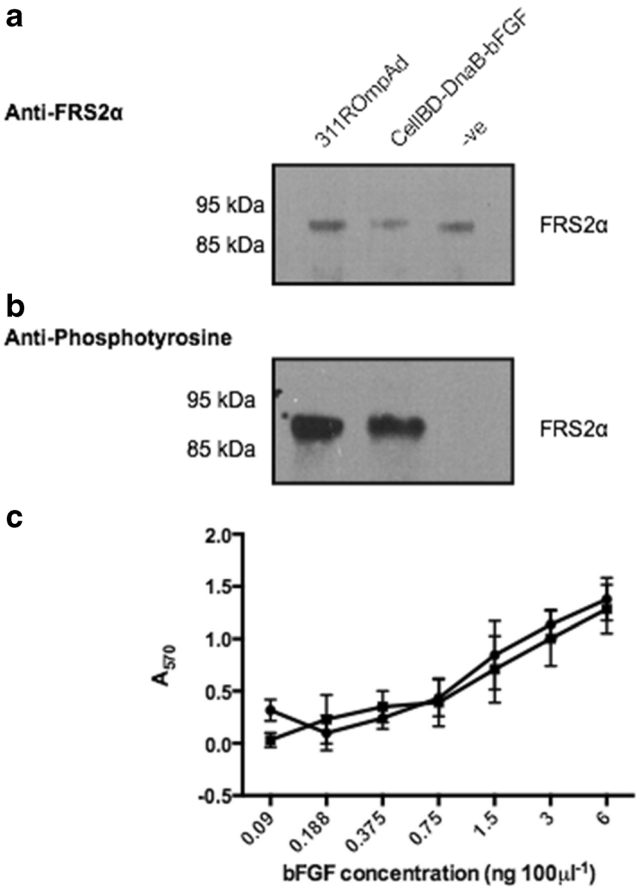

Fig. 5 Mitogenicity of recombinant bFGF. Details of purification of bFGF from cell lysates and evaluation of bFGF bioactivity were described in "Materials and methods." **a** and **b** show Western blot results with antibodies raised against FRS2α and phosphotyrosine, respectively, and the two blots contain the same arrangement of bFGF samples. Lane 311ROmpAd: bFGF purified from *E. coli* [pWK311ROmpAd] culture (Kwong et al. 2016b); lane CellBD-DnaB-FGF: bFGF purified from *B. subtilis* [pM2-CellBD-DnaB-bFGF] culture; Lane -ve: buffer without bFGF. **c** provides a summary of the mitogenic effects exhibited by different concentrations of purified bFGF samples obtained from *E. coli* [pWK311ROmpAd] (—●—) and *B. subtilis* [pM2-CellBD-DnaB-bFGF] (—■—) cultures. The assay was repeated four times and standard error bars are shown

new approach for protein expression might be achieved by either in vivo (Kwong and Wong 2013; Kwong et al. 2016a, b) or in vitro (Starokadomskyy et al. 2008; Zhang et al. 2015) methods to facilitate auto-catalytic cleavage of fusions formed between target proteins and inteins. However, the fact that the employment of *E. coli* could result in the formation of inclusion bodies (Baneyx and Mujacic 2004), plasmid curing (Fu et al. 2005, 2006; Kwong and Wong 2013), and dramatic cell death (Kurland and Dong 1996; Fu et al. 2005, 2006; Saida et al. 2006; Bentley et al. 2009) during recombinant protein expression may impose difficulties on scale-up production.

The Gram-positive bacterium, *B. subtilis*, being also well characterized, easily manipulated, and relatively cheap to grow, is the second common host of choice employed for gene expression and has been engineered to express widely different secretory proteins (Palva 1982; Ikawa et al. 1998; Ye et al. 1999; Westers et al. 2004; Kwong et al. 2013). Nevertheless, despite being employed to achieve high levels of homologous protein expression, e.g., α-amylase, which has been expressed to result in over 1 g l^{-1} of secreted product (Ikawa et al. 1998), attaining the same level of production of heterologous proteins in *B. subtilis* has been difficult. However, from another perspective, being recognized as a GRAS organism, *B. subtilis* does not consist of endotoxins as structural components. Therefore, it presents itself as an attractive host system for the expression of valuable proteins, such as those important for medical applications. Our recent success in exploiting inteins to facilitate production of authentic bFGF in *E. coli* (Kwong and Wong 2013; Kwong et al. 2016a, b) had prompted us to investigate the feasibility of using the same approach to mediate protein expression in *B. subtilis*. We were particularly interested in understanding whether the target proteins, if expression was successful, might be attainable as intracellular and properly cleaved products in *B. subtilis*.

Until now, not much is known regarding how inteins operate in *B. subtilis*. However, the results from our study of fusions engineered among CellBD, DnaB, and bFGF clearly demonstrated the success in having bFGF expressed as an intracellular, soluble, and precisely cleaved product (Fig. 2). Our findings further support the idea that the intracellular compartment, cytoplasm, of *B. subtilis*, furnished an environment possessing the required conditions, similarly as its counterpart did in *E. coli* (Kwong and Wong 2013; Kwong et al. 2016a, b), to facilitate expression of heterologous proteins as soluble and auto-catalytically processed products. Although the use of DnaB alone resulted in only weak expression of bFGF, quite unexpectedly, by adding the 11-kDa CellBD of Eng encoded by the *cenA* gene of *C. fimi* (Wong et al. 1986) to the N-terminus of DnaB, expression of bFGF was highly enhanced (Fig. 3).

The application of CellBD as an anchor to the purification of fusion proteins expressed in *E. coli* was previously reported (Greenwood et al. 1994). Thus, it was envisaged that if our study resulted unfortunately in an insoluble CellBD-DnaB-bFGF P/I, CellBD would be useful for purifying the fusion protein, followed by its cleavage with the help of in vitro manipulations (Starokadomskyy et al. 2008; Wan et al. 2011; He et al. 2015; Esipov et al. 2017). Encouragingly, the described fusion approach resulted not only in successful expression of CellBD-DnaB-bFGF in the cytoplasm, but also a precursor protein that was soluble and auto-cleavable to yield bFGF possessing the 146 aa authentic structure (Table 2; Kwong and Wong 2013; Kwong et al. 2016b) as the product.

The abovementioned results support the following interpretations. First, although there was no prior art reporting intein-mediated expression and auto-cleavages of P/I proteins in *B. subtilis*, our findings, reminiscent of similar results achieved by our group recently in *E. coli* (Kwong and Wong 2013; Kwong et al. 2016a, b), unequivocally support the potential application

of the cytoplasmic compartment of *B. subtilis* to the production of desired recombinant proteins. Second, increasing evidence from recent studies (Phan et al. 2012; Yang et al. 2013; He et al. 2016; Ng et al. 2016) and the present work supports that cytoplasmic expression is a viable approach for the production of heterologous proteins in *B. subtilis*. Although a large number of publications echoing that secretion was a preferred option for heterologous protein expression in *B. subtilis*, the fact that transportation of foreign proteins is mediated by only a small number of membrane channels (Jacobs et al. 1995; Fu et al. 2005; Nijland and Kuipers 2008; Kang et al. 2014) explains why secretion has been inefficiently employed to achieve high levels of heterologous protein expression in *B. subtilis*. Third, there was no obvious plasmid curing of pM2-CellBD-DnaB-bFGF (Figs. 3 and 4), indicating that the plasmid, even when the host cells were grown under induced and high-density cultivation (Fig. 4), enjoyed high stability within the cells. Fourth, although stationary phase cells were subjected to a rather long period, 10 h, of induced expression, they maintained highly active and resulted in productive yields of bFGF grown under both shake flask (Fig. 3) and fermentative (Fig. 4) conditions.

Although the presence of CellBD was shown to be crucial for successful expression of CellBD-DnaB-bFGF, the exact role regarding how it facilitated expression and auto-processing of P/I is not clear. In this connection, it may be interesting to address whether other protein moieties, with different sizes and aa compositions, might act as effective as CellBD did in modulating the molecular events required to release authentic bFGF from its P/I in *B. subtilis*. In *E. coli*, it has been shown that different fusions, comprising various combinations of intein and the flanking exteins, undergo expression and auto-cleavable activities with distinctly different efficiencies (Amitai et al. 2009).

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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