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ORIGINAL PAPER

Enhanced expression of engineered ACA-less β-1, 6-*N*-acetylglucosaminidase (dispersin B) in *Escherichia coli*

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Abstract β-1,6-*N*-Acetylglucosaminidase (dispersin B), which cleaves poly-ß-(1,6)-linked *N*-acetylglucosamine, is encoded by *dspB* of *Aggregatibacter actinomycetemcomitans*. To enhance the production of dispersin B, we engineered *dspB* to transcribe mRNAs devoid of the trinucleotide ACA. Transcription and translation levels of ACA-less and wild-type *dspB* expressed in *Escherichia coli* (*E. coli*) under T5 and T7 promoters were analyzed by real-time RT-PCR and protein quantification, respectively. The ACA-less *dspB* mRNA level was significantly higher ($P < 0.01$) and produced 77.6 and 34.9% more dispersin B than wild-type *dspB* expressed under T7 and T5 promoters, respectively. Dispersin B expression under T7 promoter caused a 98–99.5% drop in the glyceraldehyde-3-phosphate dehydrogenase (*gapA*) mRNA level, which was not observed with T5 promoter. Fusion of green fluorescent protein (GFP) with dispersin B allowed rapid quantification of dispersin B production by measuring fluorescence intensity in culture broth. Although the cultures containing 0.1% glucose showed sustained increase in dispersin B-GFP production until 12 h, no significant increase in dispersin B activity was observed beyond 4 and 6 h after induction when expressed under T7 and T5 promoters, respectively. This study demonstrates the effectiveness of ACA-less mRNA and the advantage of GFP tagging for enhanced dispersin B production and quantification, which could be adapted for improving the production of other commercially important proteins in *E. coli*.

Keywords Dispersin B · ACA-less sequence · MazEF · GFP tagging · *Escherichia coli*

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Introduction

β-1,6-*N*-Acetylglucosaminidase (dispersin B), a β-hexosamidase enzyme produced by *Aggregatibacter actinomycetemcomitans*, cleaves poly-β-(1,6)-*N*-acetylglucosamine (poly-β-(1,6)-GlcNAc or PGA) to generate a series of glucosamine-containing oligosaccharides [7, 11]. Dispersin B has been shown to inhibit as well as disperse biofilms of important bacterial pathogens such as *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Actinobacillus pleuropneumoniae* and *Aggregatibacter actinomycetemcomitans*, which depend upon PGA for biofilm structural stability and are known to colonize host tissues and/or medical devices [7, 8, 21]. Because of the widespread involvement of biofilm formation in many kinds of bacterial infections, dispersin B may be applicable for the treatment of a variety of device-related and wound infections. Recognizing the commercial potential of dispersin B, we expressed this protein in *E. coli* and examined a novel molecular approach to enhance the production of dispersin B. Our approach was to engineer a *dspB* gene that was devoid of the trinucleotide sequence ACA.

It has been demonstrated that the mRNA transcripts lacking "ACA" sequence are protected from MazF mediated degradation and stably co-expressed and translated in

MazF-induced cells [19, 20]. The *mazE* and *mazF* genes encode a labile antitoxin MazE and a stable toxin MazF, respectively. MazF is a sequence-specific (ACA) endoribonuclease that functions only on single-stranded RNAs [23, 24]. MazF efficiently blocks protein synthesis by cleaving cellular mRNA, leading to growth arrest and cell death [24]. The MazF effect is relieved by MazE; hence, the death or survival of the cell is decided based on the level and ratio of MazF to MazE. In our approach to optimize dispersin B production, we have examined the expression of ACA-less *dspB* mRNA without the MazF overexpression, but relied upon induction of MazF accumulation as a stress response to metabolic burden caused by overproduction of recombinant proteins in *E. coli*.

The isolation of recombinant proteins from bacterial systems often requires a laborious optimization of expression and purification conditions. To simplify this procedure, researchers have included the green fluorescent protein (GFP) in bacterial expression vectors [10, 13, 14]. The resulting GFP fusion proteins can be sensitively detected in intact bacterial cells using either a fluorescence microscope or a fluorescence plate reader. Further, this approach permits rapid optimization of the conditions for protein expression [14]. GFP tagging also enables direct monitoring of protein expression, protein stability or solubility [14]. Thus, we have constructed a reporter gene that expresses a dispersin B-GFP hybrid protein for rapid evaluation and quantification of protein expression directly in intact cells.

Materials and methods

Strains and plasmids

Escherichia coli strains XL1-Blue (Stratagene, La Jolla, CA) and Tuner(DE3)pLacI (Novagen, Madison, WI) were used for cloning and production of dispersin B, respectively. pQE60 and pET16b vector were purchased from QIAGEN (Mississauga, ON, Canada) and Novagen (Madison, WI), respectively. The plasmids pHis-DspB1 [7] and pGFPmut2 [3] were used as the source of wild-type *dspB* and *gfp* genes, respectively. Dispersin B expression plasmids pT5DspBACA$^+$, pT5DspBACA$^-$, pT7DspBACA$^+$, pT7DspBACA$^-$, pT5DspBGFP and pT7DspBGFP were constructed using standard molecular biology techniques [16].

Recombinant DNA techniques

The *pfu* DNA polymerase and restriction endonucleases were purchased from MBI Fermentas (Burlington, ON, Canada). T4 DNA ligase and shrimp alkaline phosphatase (SAP) were from New England Biolabs (Mississauga, ON, Canada) and Roche Diagnostics (Laval, QC, Canada), respectively. Synthetic oligonucleotides (Table 1) were obtained from Sigma Genosys (Oakville, ON, Canada). All enzymatic reactions were performed according to the manufacturers' instructions. *E. coli* cells were transformed by heat shock using frozen competent cells prepared with the calcium chloride method as previously described [15]. Plasmid DNA was extracted from *E. coli* using the alkaline lysis method [16].

The *dspB* gene that does not contain "ACA" sequences (ACA-less *dspB*) was synthesized by polymerase chain reaction (PCR) in a total of 27 reactions. The first PCR reaction was carried out to create a template for the following reactions by using primers dspB-R and dspB-F1, which are complimentary at their 3′ ends. Then, the 5′ end of the template was progressively extended using 25 forward primers (dspB-F2 to dspB-27). Each forward primer (dspB-F2 to dspB-F27) was used consecutively in separate PCR reactions together with reverse primer dspB-R. At the end of PCR of each reaction, 3 µl of PCR product was used as the template for the reaction immediately following it. The thermal program used for PCR reactions 1–26 consisted of one cycle of 94°C for 5 min, three cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min. The thermal program for PCR reaction 27 consisted of 1 cycle of 94°C for 5 min, 25 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min and 1 cycle of 72°C for 5 min. The PCR generated ACA-less and wild-type *dspB* genes encode identical dispersin B that also carries an additional N-terminal His tag (6× histidine) used for purification by Ni-affinity chromatography. The PCR generated ACA-less *dspB* gene was cloned into the *Nco*I and *Bam*HI sites of pQE60 and pET16b to generate pT5DspBACA$^-$ and pT7DspBACA$^-$ plasmids. The *dspB* gene of pHis-DspB1 was amplified by PCR with dspB-27 and dspB-R primers using the same thermal program that was used for synthesis of ACA-less *dspB* gene, and cloned to the *Nco*I and *Bam*HI sites of pQE60 and pET16b to generate pT5DspBACA$^+$ and pT7DspBACA$^+$ plasmids.

The plasmid pT5DspBGFP producing dispersin B-GFP fusion protein contained the *gfp* gene fused to the 3′ end of ACA-less *dspB* via a linker sequence (WAEAAAKEAA AKEAAAKEAAAKADP). The ACA-less *dspB* gene was amplified by PCR from pT5DspBACA$^-$ using FuDspB-R and DspB-27 primers. The *gfp* gene was PCR amplified from pGFPmut2 plasmid using GFP-F and GFP-R. The *Nco*I-*Bam*HI digested ACA-less *dspB* and *Bam*HI-*Hin*dIII digested *gfp* PCR products were ligated to the *Nco*I-*Hin*dIII sites of pQE60 vector to generate pT5DspBGFP plasmid. The *dspB-gfp* fusion gene of pT5DspBGFP plasmid was amplified by using the DsGFP-R and DspB-27 primers. The *Nco*I-*Bgl*II digested *dspB-gfp* PCR product was ligated into the *Nco*I-*Bam*HI sites of Pet16b vector to generate pT7DspBGFP plasmid. Recombinant plasmids were

Table 1 Primers used for gene amplification and real time RT-PCR

Primer name	Nucleotide sequence (5′–3′)
dspB-R	ataatggatcctcgagtcactcatccccattcgtcttatg
dspB-F1	gacgaaaccattcagaaaaatacgaaaagtttattggaagcggtgattcataagacgaatggggatgag
dspB-F2	cgcagcattatcgatctggggagaagatgcaaaagcgctgaaagacgaaaccattcagaaaaatacga
dspB-F3	gatggacgaaataccaaaaaccgcgtgcaaaatactcatgaaatagccggcgcagcattatcgatctgg
dspB-F4	cctttgccgccaaagatgttataaaaaattgggatcttggtgtttgggatggacgaaataccaaaaaccg
dspB-F5	gttccgaaagcttcaccaaccttctcgcaagatgccgcctttgccgccaaagatgttat
dspB-F6	gctttactgtcctgaactataattcctattatctttatattgttccgaaagcttcaccaacctt
dspB-F7	cgtgatatgcgggtcagtttgccggagttgctggcgaaaggctttactgtcctgaactataattcct
dspB-F8	gagctatgatggcgatacgcaggataaaaatgaagctgccgagcgccgtgatatgcgggtcagttt
dspB-F9	actttgagcaaatcaacccgaatattgaaattacttattggagctatgatggcgatacgca
dspB-F10	ggttgaaaacccgaatgtggaatgacggattaattaaaaatacttttgagcaaatcaacccgaat
dspB-F11	gagtttattacgtatgccaataaactatcctacttttttagagaaaaaagggttgaaaacccgaatgtggaa
dspB-F12	gcgatgaatttggttattctgtggaaagtaatcatgagtttattacgtatgccaataaactatcc
dspB-F13	gatatttttggcgatacgagtcagcattttcatattggtggcgatgaatttggttattctgtgga
dspB-F14	gctgatagtattacttttatgcaatctttaatgagtgaggttattgatatttttggcgatacgagtcag
dspB-F15	cgccaggtagatgatgaaattgatattactaatgctgatagtattacttttatgcaatctttaatgagt
dspB-F16	gtgcaaaaagatagaggggtcaagtaccttcaaggattaaaatcacgccaggtagatgatgaaattgat
dspB-F17	gaacttgatagcccgaatcatatgacggcgatctttaaactggtgcaaaaagatagaggggtcaa
dspB-F18	cgatatcaaagcctatgctaaggcaaaaggcattgagttgattcccgaacttgatagcccgaatcatatg
dspB-F19	ccttataccggaaagccattcttgagttatcggcaacttgacgatatcaaagcctatgctaaggca
dspB-F20	gcggaaaatgccgtgcagggcaaagacggtatttatattaatccttataccggaaagccattcttg
dspB-F21	cgaccatgaaaactatgcgatagaaagccatttacttaatcaacgtgcggaaaatgccgtgcag
dspB-F22	cagcctttccggcggtaattttctgcacctgcatttttccgaccatgaaaactatgcgatagaa
dspB-F23	cacccgaggtgattaaatcctttattgataccatcagcctttccggcggtaat
dspB-F24	cagaccggattaatgctggatatcgcccgccatttttattcacccgaggtgattaaatccttt
dspB-F25	gcgtaaaaggcaattccatatatccgcaaaaaaccagtaccaagcagaccggattaatgctggatat
dspB-F26	ataatccatgggccatcatcatcatcataattgttgcgtaaaaggcaattccatatatcc
dspB-27	aataatccatgggccatcatcatcatcataattgttgcg
FuDspB-R	attaatcggatccgctttcgccgccgcttctttcgccgccgcttctttcgccgccgcttctttcgccgcc gcttccgcccactcatccccattcgtcttatgaatcac
GFP-F	attaatggatccgatggtgagcaagggcgaggagctg
GFP-R	attaataagcttacttgtacagctcgtccatgccg
DsGFP-R	tataatagatcttacttgtacagctcgtccatgccgag
RT-dspB-F	tgctaaggcaaaaggcattgag
RT-dspB-R	atttcatcatctacctggcgtg
RT-gapA-F	ttctgggctacaccgaagatgacg
RT-gapA-R	aaccggtttcgttgtcgtaccagg

analyzed by restriction digestion and verified by nucleotide sequencing.

Media and growth conditions

All *E. coli* strains were cultured in Luria-Bertani (LB) medium containing 100 mg/l ampicillin and 0–4 g/l of glucose, as indicated. Bacterial cells for purification of dispersin B, fluorescent intensity measurement, dispersin B activity assay and for extraction of RNA for RT-PCR analysis were cultured in 500 ml LB media inoculated with 50 ml of inoculum cultured for 16 h in LB containing 100 mg/l ampicillin. All bacterial cultures were incubated at 37°C on a gyratory shaker at 200 rpm. Expression of plasmid-encoded genes was induced by the addition of 1 mM IPTG at 2 h after culture initiation. In addition, *E. coli* strain Tuner (DE3)pLacI carrying plasmid pT7DspBACA$^-$ was cultured in a B. Braun 30-l fermentor (B. Braun Biotech Inc., Allentown, PA) in order to assess the production of dispersin B on a commercial scale. This fermentation was carried out by BioVectra, a contract manufacturing organization (Charlottetown, PEI, Canada).

Purification and analysis of dispersin B

Dispersin B was extracted from cultures of *E. coli* Tuner (DE3)pLacI strains bearing plasmid pT5DspBACA$^+$, pT5DspBACA$^-$, pT7DspBACA$^+$, pT7DspBACA$^-$ and pT7DspBGFP or pT5DspBGFP. Bacterial cells were harvested by centrifugation at different time points as indicated. Protein purification was conducted by following the method of Kaplan et al. [11] with slight modifications. Cell pellets were resuspended in 1/25 of the original culture volume of extraction buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 1 mM PMSF, 2 mg/ml lysozyme and 0.1% Igepal, a non-ionic detergent. Cell suspensions were sonicated three times, each for 10 s at 30% capacity using the Fisher Sonic Dismembrator Model 300 (Fisher Scientific, Ottawa, ON, Canada) equipped with a microprobe and incubated on ice for 30 min. Cell lysates were supplemented with DNaseI and RNaseA to a final concentration of 5 and 10 μg/ml, respectively, and incubated at room temperature for 30 min with gentle shaking. The cell debris was pelleted by centrifugation at 13,000 rpm for 30 min at 4°C. The dispersin B was captured by passing the cleared lysate through a column of His-Select™ Nickel Affinity Gel (bed volume of ½ the lysate volume) equilibrated with three column volumes of extraction buffer. The column was washed two times with three bed volumes of extraction buffer containing 5 and 20 mM imidazole, successively. Dispersin B was eluted from Nickel Affinity Gel with two bed volumes of extraction buffer containing 100 mM imidazole. The dispersin B-containing fractions were pooled and dialyzed against 100 mM phosphate buffer (pH 5.9) containing 200 mM NaCl. Purified dispersin B was mixed with equal volumes of glycerol and stored at −20°C.

Dispersin B was visualized by SDS-PAGE with Coomassie blue staining. Protein content was determined by the BCA method [18] using bovine serum albumin as a standard protein. The dispersin B enzyme assay was carried out as described by Kaplan et al. [11] using cleared cell lysate containing total soluble protein or purified dispersin B and 4-nitrophenyl *N*-acetyl-ß-D-glucosamine as the substrate. The activity of purified dispersin B was also confirmed by testing its ability to inhibit and disperse biofilm of *Staphylococcus epidermidis* 1457 following the method of Jackson et al. [9].

Quantification of dispersin B-GFP expression

The expression of dispersin B-GFP fusion protein produced by *E. coli* Tuner(DE3)pLacI bearing the plasmids pT5DspBGFP or pT7DspBGFP was determined spectrofluorometrically by measuring the fluorescence of the culture broth. The bacterial cultures were grown in LB media containing 0–4 g/l glucose. Samples of 1 ml were taken at 2-h intervals starting soon after induction with IPTG. The samples were diluted ten times in double-distilled water, and 200 μl each per well was added to the 96-well microtiter plate. The fluorescent intensity of the cells was measured by using FLUOstar OPTIMA microplate reader equipped with FLUOstar OPTIMA software version 1.10.0 (BMG Lab Technologies Inc., Durham, NC) at excitation and emission wavelengths of 485 and 510 nm, respectively.

Real-time RT-PCR

Escherichia coli Tuner(DE3)pLacI strains bearing plasmid pT5DspBACA$^+$, pT5DspBACA$^-$, pT7DspBACA$^+$ or pT7DspBACA$^-$ were cultured without glucose. Samples of 1.7 ml were taken soon after the induction with IPTG, then at 1-h intervals for 6 h and used for RNA extraction. The RNA extraction was conducted following the methods described in Yakandawala et al. [22]. Real time RT-PCR was conducted using QuantiFast™ SYBR Green RT-PCR kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instructions in LightCycler® 2.0 Real-Time PCR System (Roche Applied Science, Laval, Quebec, Canada). The *dspB* transcripts were amplified with RT-dspB-F and RT-dspB-R primers. The *gapA* (D-glyceraldehyde-3-phosphate dehydrogenase) transcripts used as the standard were amplified with RT-gapA-F and RT-gapA-R primers. PCR products were analyzed by agarose gel electrophoresis and melting point determination.

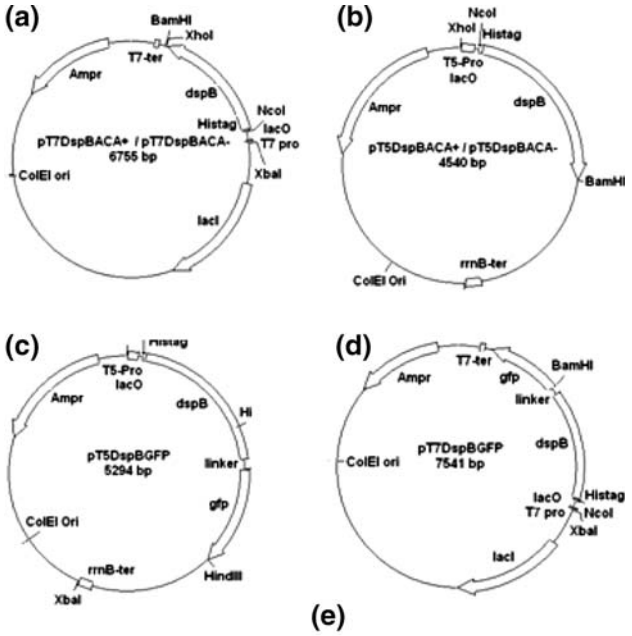
Statistical analysis

All the experiments were performed at least three times, and the results are expressed as mean ± standard deviation (SD). Statistical analysis was performed with Student's *t* test. *P* values of <0.01 were considered statistically significant.

Results

RT-PCR analysis of *dspB* transcription

We compared the mRNA level of the wild-type and ACA-less *dspB* genes transcribed under T5 and T7 promoters from plasmids pT5DspBACA$^+$, pT5DspBACA$^-$, pT7DspBACA$^+$ and pT7DspBACA$^-$ (Fig. 1a, b). ACA-less *dspB* was constructed through silent mutagenesis by substituting one nucleotide in each of the ACA sequences in the wild-type gene (Fig. 1e). Transcript levels of *dspB* and *gapA* genes were analyzed by a real-Time RT-PCR. The expression under the T5 promoter showed a rapid increase in relative *dspB* transcript levels (Fig. 2f), followed by a lag



(e)

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aat tgt tgc gta aaa ggc aat tcc ata tat ccg
caa aaa acc agt acc aag cag acc gga tta atg
ctg gat atc gcc cgc cat ttt tat tca ccc gag
gtg att aaa tcc ttt att gat acc atc agc ctt
tcc ggc ggt aat ttt ctg cac ctg cat ttt tcc
gac cat gaa aac tat gcg ata gaa agc cat tta
ctt aat caa cgt gcg gaa aat gcc gtg cag ggc
aaa gac ggt att tat att aat cct tat acc gga
aag cca ttc ttg agt tat cgg caa ctt gac gat
atc aaa gcc tat gct aag gca aaa ggc att gag
ttg att ccc gaa ctt gat agc ccg aat cat atg
acg gcg atc ttt aaa ctg gtg caa aaa gat aga
ggg gtc aag tac ctt caa gga tta aaa tca cgc
cag gta gat gat gaa att gat att act aat gct
gat agt att act ttt atg caa tct tta atg agt
gag gtt att gat att ttt ggc gat acg agt cag
cat ttt cat att ggt ggc gat gaa ttt ggt tat
tct gtg gaa agt aat cat gag ttt att acg tat
gcc aat aaa cta tcc tac ttt tta gag aaa aaa
ggg ttg aaa acc cga atg tgg aat gac gga tta
att aaa aat act ttt gag caa atc aac ccg aat
att gaa att act tat tgg agc tat gat ggc gat
acg cag gat aaa aat gaa gct gcc gag cgc cgt
gat atg cgg gtc agt ttg ccg gag ttg ctg gcg
aaa ggc ttt act gtc ctg aac tat aat tcc tat
tat ctt tat att gtt ccg aaa gct tca cca acc
ttc tcg caa gat gcc gcc ttt gcc gcc aaa gat
gtt ata aaa aat tgg gat ctt ggt gtt tgg gat
gga cga aat acc aaa aac cgc gtg caa aat act
cat gaa ata gcc ggc gca gca tta tcg atc tgg
gga gaa gat gca aaa gcg ctg aaa gac gaa acc
att cag aaa aat acg aaa agt tta ttg gaa gcg
gtg att cat aag acg aat ggg gat gag tga
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Fig. 1 Maps of dispersin B expression plasmids and the nucleotide sequence of *dspB*. **a** Plasmid expressing wild-type (pT7DspBACA+) or ACA-less *dspB* (pT7DspBACA−) gene under the T7 promoters. **b** Plasmid expressing wild-type (pT5DspBACA+) or ACA-less *dspB* (pT5DspBACA−) gene under the T5 promoter. **c** Plasmid expressing ACA-less *dspB* and *GFP* gene fusion under the T5 promoter (pT5DspBGFP). **d** Plasmid expressing ACA-less *dspB* and *GFP* gene fusion under the T7 promoter (pT7DspBGFP). **e** Nucleotide sequence of ACA-less *dspB*. The ACA sequences are *underlined*, and the substituted nucleotides are in *bold case*

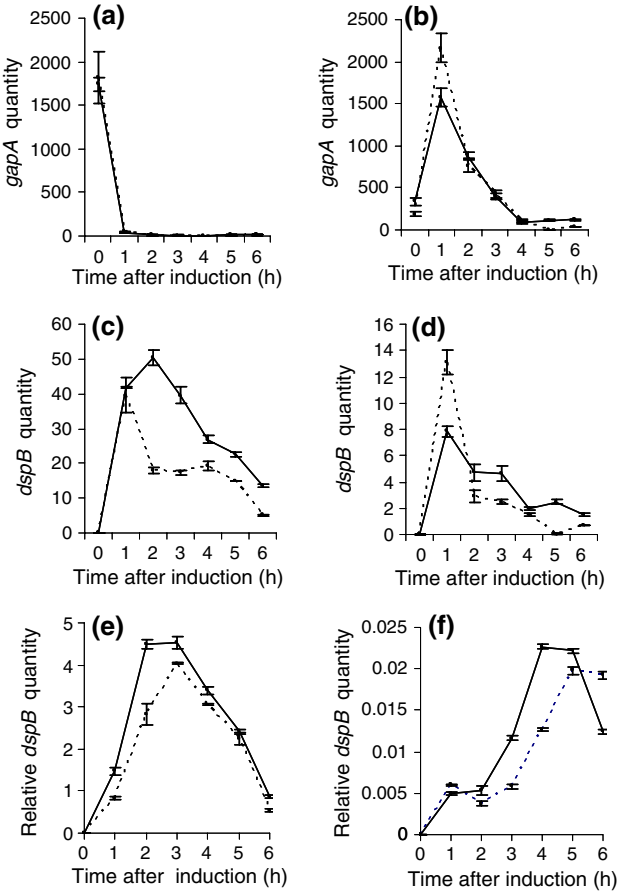

Fig. 2 RT-PCR analysis of *dspB* mRNA levels in dispersin B expressing *E. coli*. Transcript level of *gapA* gene was used as a standard reference. Graphs (**a**) and (**b**) show the *gapA* mRNA quantities in *E. coli* overexpressing wild-type (*dashed line*) and ACA-less (*solid line*) *dspB* under T7 and T5 promoters, respectively. Graphs (**c**) and (**d**) show the *dspB* mRNA quantities in *E. coli* overexpressing wild-type (*dashed line*) and ACA-less (*solid line*) *dspB* under the T7 and T5 promoters, respectively. Graphs (**e**) and (**f**) show the relative *dspB* mRNA quantities (*dspB* quantity/*gapA* quantity) in *E. coli* overexpressing wild-type (*dashed line*) and ACA-less (*solid line*) *dspB* under the T7 and T5 promoters, respectively. Each data point represents the mean value of three replicates ± standard deviation (SD), which is indicated by an *error bar*

phase, an exponential phase and another lag phase leading to a sharp decline in the transcript levels. The transcription under T7 promoter showed an exponential increase in the relative *dspB* mRNA level (Fig. 2e). The ACA-less *dspB* mRNA level was significantly higher ($P < 0.01$) as compared to that of wild-type *dspB* mRNA at all time points except at 1 h after IPTG induction under both promoters (Fig. 2c, d). The *dspB* mRNA level of ACA-less *dspB* reached the maximum earlier than that of wild-type *dspB* under the T7 promoter (Fig. 2c). The wild-type *dspB* and ACA-less *dspB* expressed under the T7 promoter produced

up to 755-fold and 840-fold more relative *dspB* mRNA, respectively, as compared to that under the T5 promoter at different time points (Fig. 2e, f). The increase in the level of ACA-less transcription under the T7 promoter caused a 97-98% decrease in *the gapA* mRNA level during the 0–1-h period after induction followed by a further 2.4% drop during the 1–6-h period (Fig. 2a). In the cells expressing *dspB* under T5 promoter, both *gapA* and *dspB* transcript levels showed a similar pattern, wherein the *gapA* mRNA levels showed a sharp increase (367%) during 0–1 h followed by 92% gradual decrease during the 1–6 h period (Fig. 2b).

Evaluation of dispersin B protein expression under two promoter systems

The dispersin B production from ACA-less and wild-type *dspB* genes under the transcriptional control of T5 (pT5DspBACA$^-$ and pT5DspBACA$^+$) and T7 (pT7DspBACA$^+$ and pT7DspBACA$^-$) promoters were compared. Both wild-type and ACA-less *dspB* genes used in this study contained an additional sequence-encoding His tag at the 5′ end to aid in the purification of dispersin B using Ni-affinity gel. Dispersin B was purified from bacterial cells grown in 500 ml LB medium containing 0.1% glucose and harvested at 4 h after IPTG induction. While ACA-less *dspB* compared to wild-type *dspB* generated 1.8-fold (236.1 vs. 132.9 mg/l, $P < 0.01$) more dispersin B under the expression signals of the T7 promoter, no significant difference between the ACA-less and wild-type in dispersin B production (59.8 vs. 44.3 mg/l) was observed when expressed under the T5 promoter. Furthermore, a trial fermentation run in a 30-l fermentor without optimizing the conditions showed an approximately seven-fold increase in the production of purified dispersin B as compared to that in a 2-l shake flask culture (544 vs. 79 mg/l).

Direct quantification of dispersin B expression using GFP tag

In order to quantify the expression of dispersin B directly in the culture broth containing intact cells, we constructed the recombinant plasmid pT7DspBGFP (Fig. 1d) expressing ACA-less dispersin B-GFP fusion protein under the T7 promoter. The dispersin B-GFP expression was quantified spectrofluorometrically by measuring the fluorescence intensity of culture broth samples. We examined the expression of dispersin B-GFP in the shake-flask cultures with the media containing different glucose concentrations (0–0.4%) and induced with 1 mM IPTG. The expression of dispersin B-GFP under the T7 promoter in media containing 0, 0.1 and 0.2% glucose reached the peak at 8, 12 and 10 h (after induction), respectively (Fig. 3). A significantly higher ($P < 0.01$) dispersin B-GFP expression was

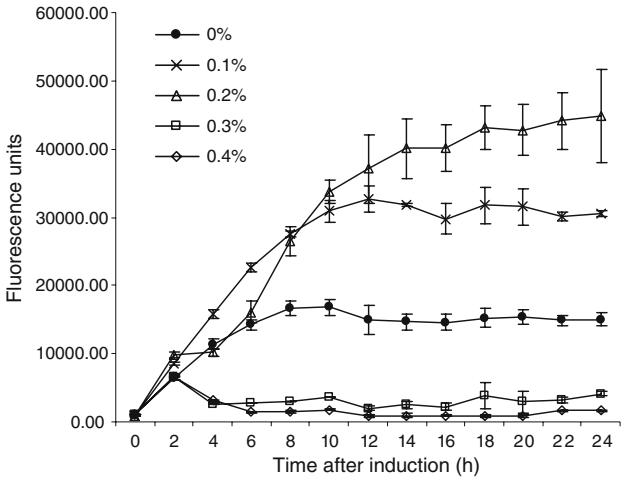

Fig. 3 Effect of glucose concentration on dispersin B production. The *E. coli* cells harboring ACA-less *dspB-GFP* gene fusion construct (pT7DspBGFP) were grown in LB media containing 0–0.4% glucose. The expression level of dispersin B-GFP was measured by fluorescence intensity at 2-h intervals following induction. The data shown are the mean values of three replicates ± standard deviation (SD), which is indicated by an *error bar*

observed in the culture broth containing 0.1% glucose at both 4 and 6 h after induction. Dispersin B-GFP production in the culture broth containing 0.1 and 0.2% glucose was similar at 2 and 8 h after induction. The mean fluorescent intensity during the 2–12-h period after induction and the corresponding dispersin B-GFP production under the T7 promoter in the culture broth containing different glucose levels was in the following order: 0.1% > 0.2% > 0 > 0.3% > 0.4%. We also monitored the expression of dispersin B-GFP in response to induction with four different IPTG concentrations (0.25, 0.5, 1 and 2 mM) in culture broth containing 0.1 and 0.2% glucose during the period of 0–12 h after induction. There was no significant difference of GFP fluorescence observed among the four different IPTG concentrations tested at each glucose level (data not shown).

Determination of optimal harvest time

The production and stability of dispersin B in shake-flask cultures producing dispersin B-GFP were studied by monitoring the fluorescence intensity and the dispersin B enzyme activity, respectively, in cleared cell lysate containing total soluble proteins. Samples were taken at 2-h intervals after induction with IPTG from the cultures of *E. coli* Tuner(DE3)pLacI bearing pT5DspBGFP (Fig. 1c) and pT7DspBGFP (Fig. 1d) grown in media containing 0.1% glucose. The expression of dispersin B-GFP under both T5 (pT5DspBGFP) and T7 (pT7DspBGFP) promoters as determined by the fluorescence intensity increased until

12 h after induction. However, dispersin B enzyme activity did not follow the same increasing trend as dispersin B-GFP production beyond 4 h, indicating inactivation or degradation of dispersin B. While the activity of dispersin B expressed under the T5 significantly increased (Fig. 4a, $P > 0.01$) until 6 h after induction, dispersin B expressed under the T7 promoter increased until 8 h (Fig. 4b). However, the dispersin B activity at 6, 8, 10 and 12 h was not significantly different from that of 4 h after induction. Thus, the optimal time for harvesting dispersin B appeared to be between 4 and 6 h after induction. Though the activity of dispersin B was significantly higher under the T7 promoter compared to that under the T5 promoter at all time points, there was no noticeable difference in the fluorescent intensity (Fig. 4a, b). This is possibly due to the differential degradation of the ACA sequence containing *gfp* of *dspB-gfp* mRNA.

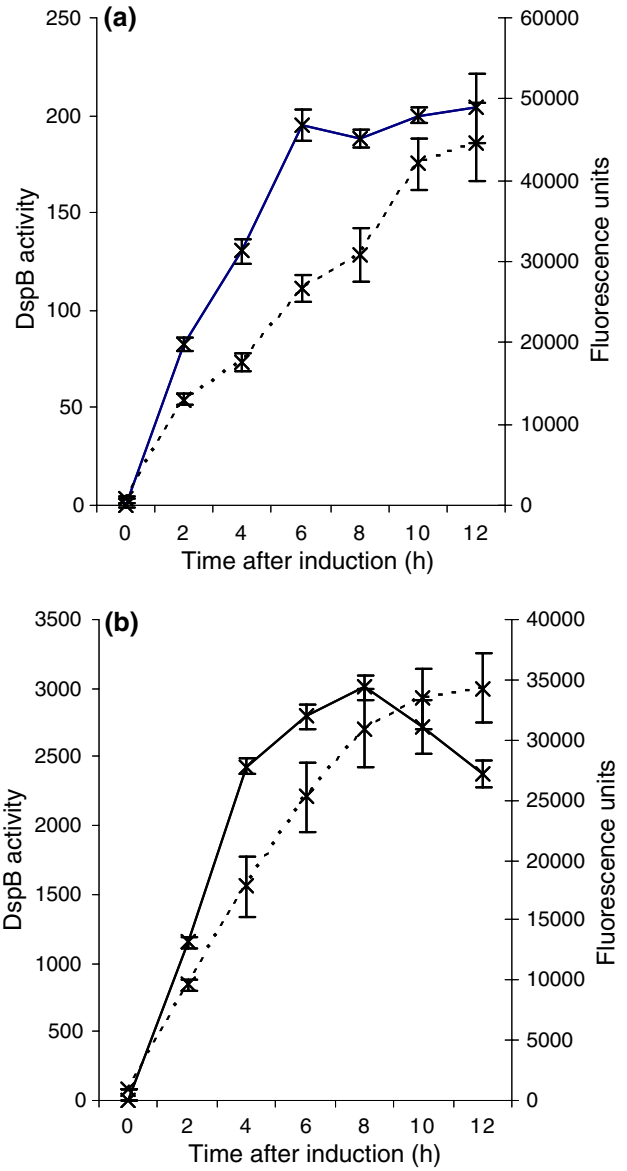

Fig. 4 Evaluation of the expression level and activity of dispersin B. The *E. coli* cells were cultured in media containing 0.1% glucose. The expression level of dispersin B-GFP was measured by fluorescence intensity (*dashed line*). The enzymatic activity (*solid line*) of dispersin B is presented as μM of nitrophenol produced per min per ml of cleared cell lysate containing total soluble protein. (**a**) and (**b**) represent the ACA-less *dspB-GFP* fusion gene constructs transcribed by T5 (pT5DspBGFP) and T7 (pT7DspBGFP) promoters, respectively. The data shown are the mean values of three replicates ± standard deviation (SD), which is indicated by an *error bar*

Discussion

This study examined a novel genetic approach to enhance the production of recombinant dispersin B in *E. coli* by engineering the *dspB* gene to produce ACA-less mRNA transcripts. Furthermore, an additional increase in dispersin B production was obtained through partial optimization of shake-flask fermentation conditions using GFP as a reporter with an ACA-less *dspB-gfp* fusion construct. The production of a significantly higher quantity of ACA-less *dspB* mRNA transcripts as compared to that of wild-type *dspB* was observed in this study. This apparently was due to the protection of ACA-less transcripts from degradation by MazF. In *E. coli*, the metabolic burden due to the overexpression of heterologous proteins activates stress responses and the corresponding changes in gene expression [2, 6]. The expression of *mazEF* has been shown to be elevated under stress conditions such as amino acid starvation [1] and the presence of antibiotics that are known to inhibit transcription and translation [12, 17]. The expression of nucleotide 3′,5′-bispyrophosphate (ppGpp), a regulatory signal molecule that exerts a toxic effect on cells, has been shown to be elevated in *E. coli* cells overproducing recombinant proteins [4, 5]. Furthermore, the cytotoxic effect of ppGpp has been shown to be mediated through activation of MazF [1]. A single protein production (SPP) system that exploits ACA-specific endoribonuclease activity of MazF has been utilized for increasing the production of a protein from a target gene lacking the "ACA" sequence [19, 20]. Also, a significant improvement in recombinant protein synthesis in *E. coli* has been achieved by the elimination of intracellular ppGpp synthesis [5], presumably by the stabilization of target mRNA transcripts containing ACA sequences.

In this study, we anticipated a consequential increase in ppGpp synthesis in *E. coli* upon induction of recombinant dispersin B production, with downstream effects on *mazEF* expression. Our studies suggested that greater dispersin B production from the T7 promoter system as compared to the T5 was due to a much greater amount of *dspB* transcription in the former system. Thus, it appears likely that the metabolic burden imposed by a higher rate of *dspB* mRNA

transcription from the T7 promoter, specifically during the first hour after induction, caused the observed rapid decrease in *gapA* mRNA due to MazF-dependent degradation. The observed increase in dispersin B production from ACA-less *dspB* in comparison to the wild-type *dspB* gene was apparently due to higher steady state levels of ACA-less *dspB* mRNA under both T5 and T7 promoter systems. However, the effect of ACA-less *dspB* on dispersin B production was significantly higher ($P < 0.01$) in cells harboring the T7 promoter system, which could not only be due to an enhanced MazF activity resulting in the degradation of other cellular mRNAs competing for protein synthesis, but also due to an increased transcription of *dspB*.

Furthermore, we expressed ACA-less *dspB* as dispersin B-GFP fusion protein in *E. coli*. The GFP tag was used for immediate visualization and quantification of fusion protein expression. Monitoring of the fluorescence intensity and the dispersin B activity over a period of time allowed us to understand the rate of dispersin B production and the onset of product degradation and/or inactivation. It is possible that the fluorescence of dispersin B-GFP has been retained even after the dispersin B part is degraded or inactivated. Despite the continuous synthesis of dispersin B-GFP, as indicated by the increase in the fluorescence intensity over a 12-h period, the activity of dispersin B did not seem to improve beyond 4 h after induction when expressed under both the T7 and T5 promoters. These results indicate the possible degradation or loss of activity of dispersin B when the shake-flask fermentation time was extended, which helped us to determine the optimal time of harvesting dispersin B.

Our findings suggest that it may be feasible to produce other recombinant proteins on a commercial scale using an *E. coli* expression system with ACA-less target gene sequences, in the absence of MazF overproduction, provided that transcription of the target gene is active enough to cause a metabolic burden capable of triggering endogenous MazF via ppGpp accumulation. Also, the seven-fold increase in dispersin B production in a 30-l fermentor using partially optimized shake-flask parameters showed the potential of *E. coli* strain overexpressing ACA-less *dspB* for commercial scale production of dispersin B under optimized fermentation conditions. In addition, this study demonstrates the versatility of the GFP tagging technique in the optimization of fermentation conditions for enhanced production of dispersin B and other proteins of commercial importance in *E. coli*.

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