Electronic Journal of Biotechnology 42 (2019) 49–55

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Twin-arginine signal peptide of *Bacillus licheniformis* GlmU efficiently mediated secretory expression of protein glutaminase

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ARTICLE INFO

Article history:
Received 12 May 2019
Accepted 18 October 2019
Available online 26 October 2019

Keywords:
Arginine
Aspartic acid
Bacillus licheniformis
Bacillus subtilis
Escherichia coli
Prostaglandins A
Protein glutaminase
Protein sorting signals
Secretion expression
Signal peptide
Tat pathway

ABSTRACT

Background: Protein glutaminase specifically deamidates glutamine residue in protein and therefore significantly improves protein solubility and colloidal stability of protein solution. In order to improve its preparation efficiency, we exploited the possibility for its secretory expression mediated by twin-arginine translocation (Tat) pathway in *Bacillus licheniformis*.
Results: The *B. licheniformis* genome-wide twin-arginine signal peptides were analyzed. Of which, eleven candidates were cloned for construction of expression vectors to mediate the expression of *Chryseobacterium proteolyticum* protein glutaminase (PGA). The signal peptide of GlmU was confirmed that it significantly mediated PGA secretion into media with the maximum activity of 0.16 U/ml in *Bacillus subtilis* WB600. A mutant GlmU-R, being replaced the third residue aspartic acid of GlmU twin-arginine signal peptide with arginine by site-directed mutagenesis, mediated the improved secretion of PGA with about 40% increased (0.23 U/ml). In *B. licheniformis* CBBD302, GlmU-R mediated PGA expression in active form with the maximum yield of 6.8 U/ml in a 25-l bioreactor.
Conclusions: PGA can be produced and secreted efficiently in active form via Tat pathway of *B. licheniformis*, an alternative expression system for the industrial-scale production of PGA.
How to cite: Niu D, Li C, Wang P, et al. Twin-arginine signal peptide of *Bacillus licheniformis* GlmU efficiently mediated secretory expression of protein glutaminase. Electron J Biotechnol 2019;42. https://doi.org/10.1016/j.ejbt.2019.10.006

1. Introduction

Protein glutaminases (EC 3.5.1.44) are a kind of enzymes that specifically hydrolyse the amido group on glutamine residues located at the surface of proteins and therefore improve protein solubility and colloidal stability of protein solution [1]. Their representative is *Chryseobacterium proteolyticum* protein glutaminase (PGA), which was discovered and biochemically identified in 2000 [2,3]. The PGA was encoded by a 1053 bp open reading frame and synthesized as a prepro-form, using a 21-amino-acid signal peptide, a 114-amino-acid pro-region (MW = 12,848.2), and a 185-amino-acid mature enzyme (MW = 19,856.6). The PGA with its pro-region had no enzymatic activity [3].

Since the wild strain of *C. proteolyticum* produced too small amount of PGA to be suitable for industrial applications [3], its heterologous over-expression is an undoubtedly way to be exploited. For this purpose, in previous researches, PGA over-expressed using the Tat pathway in *Corynebacterium glutamicum* has been investigated [4]. It was found that PGA could be produced and secreted efficiently in an inactive pro-enzyme form in *C. glutamicum* mediated by a Tat-dependent signal peptide from *Arthrobacter globiformis* IMD [5,6] or *Escherichia coli* TorA [6,7]. This formed inactive pro-PGA could subsequently be converted to an active form by a subtilisin-like serine protease, SAM-P45 [8]. The maximal accumulation of pro-PGA in *C. glutamicum* reached up to 183 mg/l (about 4.8 U/ml) [4]. These results clearly indicated that the Tat pathway in bacteria could be an alternative for the industrial scale preparation of PGA [4,6,9].

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2019.10.006

Remarkably, some strains from *Bacillus licheniformis* have been used for industrial scale enzyme production for over 50 years because of their dramatically high capacity of enzyme synthesis and secretion (more than 25 g/l) [10]. However, their Tat pathways were not well investigated and exploited for the production of enzymes.

In this report, we examined the possibility for high efficiency production of PGA in *B. licheniformis*. The putative twin-arginine signal peptides in *B. licheniformis* were genome-widely selected and functionally identified. The twin-arginine signal peptide of GlmU was found to efficiently mediate the secretion of PGA, and the PGA in active form could be efficiently produced in *B. licheniformis*.

2. Materials and methods

2.1. Strains, plasmids, and culture medium

The plasmids used in this study are listed in Table 1. The nucleotide sequences used for amplification of the relative signal peptide regions are listed in Table 2. *E. coli* JM109 was used for plasmid construction. *B. licheniformis* ATCC 14580 was used as a source for the selection of twin-arginine signal peptides. *Bacillus subtilis* WB600 [11] and *B. licheniformis* CBBD302 [10] were used as the expression hosts. All strains were grown in Luria–Bertani (LB) broth [12] except other mentioned. Kanamycin (20 μg/ml for *E. coli* and *Bacillus* sp.) or tetracycline (25 μg/ml for *Bacillus* sp.) was added to culture media when required. For PGA production, LB broth supplemented with 2% lactose was used as the basic broth. When necessary, another 8% lactose was fed during fermentation in 25-l bioreactor.

Table 2
Primers used in this study.

Tat signal peptide candidates and others	Primers (5'➔3′)[a]
GlmU	P_{GlmU}-1: ATGGATAATAGGGATAATGGAGGC
	P_{GlmU}-2: cctggatccAGGATGAAGAACTTTATATAGCTT TGA
YxaJ	P_{YxaJ}-1: ATGAAAACCAATAGAGTAAGTGTAATGATCT
	P_{YxaJ}-2: ctcggatccGAGCAAAACGGTTCCGATTG
YbxG	P_{YbxG}-1: ATGGCGAACAAAGAATTGAAGA
	P_{YbxG}-2: atcggatccGATAAAAATTCCGCAAATGGC
YbgF	P_{YbgF}-1: ATGGAACAGTCAAACACCAATCGCC
	P_{YbgF}-2: gtcggatccGACGATCAGAGCCCCGGC
PhoD	P_{PhoD}-1: ATGAAAAAACTGAGCGAGGAAAGCC
	P_{PhoD}-2: gatggatccATCTCCCGATGCAACGCCAAG
YcgH	P_{YcgH}-1: ATGAAACGACAAGCGAAAAAAGGAG
	P_{YcgH}-2: actggatccAGTGCCAAGGGCAGCAAGAAG
YdbS	P_{YdbS}-1: ATGAGAAGCGAGCCGAAAAATCAAA
	P_{YdbS}-2: gcgggatccCGCTGACAAAATGCCGATCCA
YhdH	P_{YhdH}-1: ATGAGCGACCGCAGAATGACAG
	P_{YhdH}-2: ataggatccTATCGTAAAT AGGACAAACA CAAG GAAAAA
YhjN	P_{YhjN}-1: ATGATATTGATCAGCAGTTTTGGGG
	P_{YhjN}-2: ctaggatccTAGCATTTGGCCGAACTGAAGC
AbnA	P_{AbnA}-1: ATGTTAAAGACATCGAAATTTGAAAGGAG
	P_{AbnA}-2: attggatccAATAATGTTGTCTCCTTTTGTATCCCA
YesL	P_{YesL}-1: ATGATTCACACGTTGGCAAATGG
	P_{YesL}-2: gtcggatccGACATCCGTTTTTCCCAGCGT
pHY-WZX	Primer A: GATTCTCCTCCCCTTTCAATG
	Primer B: tctggatccAGAATTCGAGCTCCCGGGTACCAT
pro-PGA	Pga1: cgcggatcccTTGCGAGCGTTATCCCGGA
	Pga2: TTAGAAGCCGCAGCTGCTAACG
GlmU-K	P_{GluM}-K: ATGGATAAAAGGGATAATGGAGGC
GlmU-R	P_{GluM}-R: ATGGATAGGAGGGATAATGGAGGC

[a] The single underlines were *Bam*HI site added; the double underlines were mutation sites.

Table 1
Plasmids used in this study.

Plasmid	Tat signal peptide	Genotypes/Tat signal peptide nucleotide sequence and cloning site[a]	Reference
pHY-WZX	/	*amyL* promoter and signal peptide, Km^R, Tet^R	[13]
pUC-PGA	/	pUC18 containing synthesized pro-PGA encoded gene	This study
pHY-TAT1	GlmU	ATGGATAATAGGGATAATGGAGGCCAATACATGGATAAGCGGTTTGCAGTTGTGTTAGCAGCTGGTCAAGGAACAAGAATGAAA TCAAAGCTATATAAAGTTCTTCATCCTggatcc	This study
pHY-TAT2	YxaJ	ATGAAAACCAATAGAGTAAGTGTAATGATCTGGACTTTAATCTCCGCTTTTCTGTTTTGTTCTATGATAGTCGCAGCTTCACTTTCTCC GCTCGCTCATTCAGGCCCTCATGCTAATGAGTTTGGCACTTTGGGGATGTGGGCGGCAATCGGAACCGTTTTGCTCggatcc	This study
pHY-TAT3	YbxG	ATGGCGAACAAAGAATTGAAGAGAGGCCTGGGCGCGCGCCACATCCAAATGATTGCGCTTGGCGGCACTATCGGCGTCGGTTTATT TATGGGATCTTCCAGCACGATAAAGTGGACAGGACCTTCCGTCCTGCTTGCTTATGCCATTTGCGGAATTTTTATCggatcc	This study
pHY-TAT4	YbgF	ATGGAACAGTCAAACACCAATCGCCAGAACTTTCAAAGAAAAATGCAAACGAGACACCTTATCATGCTTTCCTTAGGAGGCGTTAT CGGCACGGGCCTTTTCTTAAGTTCCGGTTATACGATCTCACAGGCTGGTCCTGCCGGAACGATTCTAGCCTACTTGGCCGGGGCT CTGATCGTCggatcc	This study
pHY-TAT5	PhoD	ATGAAAAAACTGAGCGAGGAAAGCCTCAAGGACAATACGTTTGACCGCCGCCGCTTTATTCAAGGGGCCGGCAAAATAGCCGGG CTTTCGCTCGGACTTGCGATCGCGCAATCGATGGGGGCAATGGAAGTCAATGCAGCACCGAGGTTCTCCGAATATCCGTTTACACTT GGCGTTGCATCGGGAGATggatcc	This study
pHY-TAT6	YcgH	ATGAAACGACAAGCGAAAAAAGGAGATTTAAAATGGTGGCAGCTGTCCATGATCGGGGTCGGCTGCACAATTGGAACGGGGTTT TTCCTCGGCTCAAGCATCGCGATTAAGAAAAGCGGCTTTTCCGTATTAGCCGCCTTTCTTCTTGCTGCCCTTGGCACTggatcc	This study
pHY-TAT7	YdbS	aTGAGAAGCGAGCCGAAAAATCAAATCAGCCGTGATGGCGTTAAAGTCTGGAGAATCACGGCTTGTATCACATCATTCGTTTTAATG CTGGCTGCAGCAGGCCTGATTGCCGCCGGGGTGATATTTAAGTGGCCGGTATGGATCGGCATTTTGTCAGCGggatcc	This study
pHY-TAT8	YhdH	ATGAGCGACCGCAGAATGACAGCAAAGTGGGCATCCAAACTTGGATTTGTTCTCGCCGCCGCGGGGTCCGCCATCGGTCTGGGAGC GATTTGGAAGTTTCCCTATGTGGCGGGAACGAGCGGGGGAGGCGCATTTTTCCTTGTGTTTGTCCTATTTACGATAggatcc	This study
pHY-TAT9	YhjN	aTGATATTGATCAGCAGTTTTGGGGGATTTCTTCTCTCATTGACCGGCATGACAATCGGCTGGATGGTCGGAACAATGACCGCCGCC GCTTGTCTGTCGCTGTTTCGCCCGTCACCGTTGAAAGCGGCGGTCAGACAAAACGGCATCCACCATGGATGGCTTCAGTTCGGCC AAATGCTAggatcc	This study
pHY-TAT10	AbnA	ATGTTAAAGACATCGAAATTTGAAAGGAGAACAACTGTGGCTAAGACTATCATTTCTGGTTTTATTTTATTTTTTCTGCTTATTTTCTC GGCGGCTGAACCAACATCAGCGGCTTTTTGGGATACAAAAGGAGACAACATTATTggatcc	This study
pHY-TAT11	YesL	ATGATTCACACGTTGGCAAATGGATTTTACCGCTTTTGCGAGTGGGTGATGCGGCTTGCCTATTTGAATCTGCTGTGGATCGGCTT TACGCTGGCGGGAGCGGTCATCTTCGGTTTAGCGCCGGCGACCGCCGCGATGTTCGCTGTGACTAGACAGTGGACGCTGGGAAAA ACGGATGTCggatcc	This study
pHY-TAT1r	GlmU-R	Derived from pHY-TAT1 in which the third codon for aspartic acid was changed for arginine	This study
pHY-TAT1k	GlmU-K	Derived from pHY-TAT1 in which the third codon for aspartic acid was changed for lysine	This study
pHY-PGA1	GlmU	PGA-encoded gene in pHY-TAT1	This study
pHY-PGA1r	GlmU-R	PGA-encoded gene in pHY-TAT1r	This study
pHY-PGA1k	GlmU-K	PGA-encoded gene in pHY-TAT1k	This study

[a] Underlined were *Bam*HI site added.

2.2. DNA manipulations

DNA manipulations were performed using conventional techniques [12]. Polymerase chain reactions (PCR), with Pyrobest DNA polymerase (TakaraBio, Dalian, China), were performed in 50 μl working volumes and DNA products were recovered from the agarose gels with the QIAquick gel extraction kit (Qiagen, Germany). Nucleotide sequences were determined using a BigDye terminator cycle sequencing kit (Applied Biosystems) and a DNA Sequencer (model 3200; Applied Biosystems). Plasmid transformations into *E. coli*, *B. subtilis* or *B. licheniformis* were carried out according to the methods as described previously [13].

2.3. Construction of a set of plasmids for pro-PGA secretion using various signal peptides from B. licheniformis

When constructing a set of plasmids for pro-PGA secretion, the Tat signal peptide sequence was recovered by PCR with primers listed in Table 2 from *B. licheniformis* ATCC 14580 genome. The signal peptide region in pHY-WZX [13] was then replaced with each of putative twin-arginine signal peptide sequence by *Bam*HI-digested PCR product of pHY-WZX(B-) using Primer A and Primer B (Table 2) and *Bam*HI-digested Tat signal peptide PCR products to yield expression plasmids, pHY-TATs (Fig. 1). For pro-PGA expression, the codon optimized encoding sequence for the pro-PG based on its published amino acid sequence with GenBank accession no. AB046594 was synthesized and cloned into pUC18 plasmid to yield pUC-PGA. The synthesized gene encoding pro-PGA was then recovered by PCR with primers Pga1 and Pga2 (Table 2). It was then digested with *Bam*HI and cloned into each of pHY-TAT plasmids to yield pro-PGA expression vectors, pHY-PGAs.

Table 3
Amino acid residues around putative SPase I cleavage sites[a]

-3		-1		+1	
Residue	Frequency (% of total)	Residue	Frequency (% of total)	Residue	Frequency (% of total)
A	39.7	A	83.8	A	38.2
S	10.3	K	8.8	K	10.3
V	7.4	G, F, P, T, M	1.5*5	G	8.8
R	5.9			N, L	7.4 × 2
L, Q, E, G	4.4 × 4			I	4.4
T, I, K, M, P	2.9 × 5			S, V, Y, P, R	2.9 × 5
Y, N, F	1.5 × 3			E, Q, F, H, T, W	1.5 × 6

[a] The frequency of a particular amino acid at the indicated positions around SPases I cleavage sites is given as the percentage of the total number of predicted signal peptides in which it appears.

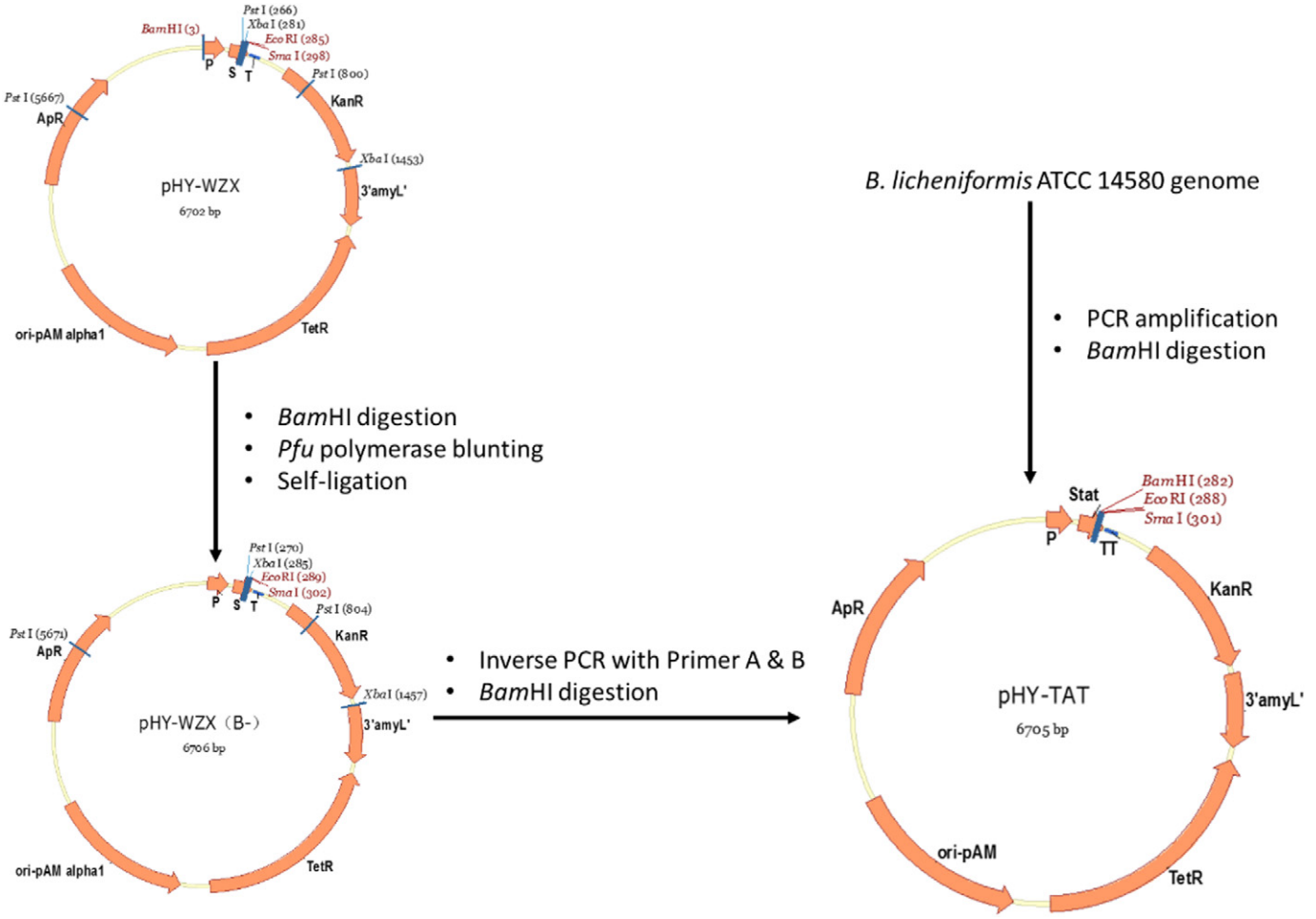

Fig. 1. The construction flowchart of pHY-TAT expression plasmids. A set of plasmids containing 11 *B. licheniformis* twin-arginine signal peptides were constructed based on pHY-WZX. The *Bam*HI site in pHY-WZX was first removed and an intermediate plasmid pHY-WZX(B-) was obtained. The *amyL* signal peptide was then removed by inverse PCR and a new *Bam*HI site was introduced with Primer B. The putative twin-arginine signal peptides were recovered from *B. licheniformis* ATCC 14580 genome by PCR and a *Bam*HI site at 3′-terminal was introduced with primers. Both PCR products were digested with *Bam*HI and ligated. The ligation mixture was transformed into *E. coli* JM109 and recombinant plasmids were confirmed by restriction pattern and further by nucleotide sequencing. The constructed plasmids were nominated as pHY-TAT1, pHY-TAT2, and so on.

The site-directed mutagenesis was carried out based on PCR technique as described above. The oligonucleotides primers P_{GlmU}-K and P_{GlmU}-R (Table 2) were used to mediate mutagenesis. P_{GlmU}-K and P_{GlmU}-2 or P_{GlmU}-R and P_{GlmU}-2 were used to amplify GlmU coding sequence using plasmid pHY-PGA1 as template. The *Bam*HI-digested PCR products were ligated with the reverse PCR product of pHY-WZX (B-) followed by inserting the pro-PGA encoding sequence as described above (Fig. 1) to yield the recombinant expression plasmid pHY-PGA1k and pHY-PGA1r.

2.4. Fermentation test

For selecting candidate Tat signal peptide, the transformants were incubated at 37°C and 220 rpm in 250-ml shaking flask with working

Table 4
Eleven Tat signal peptide candidates from *B. licheniformis* ATCC 14580.

ORFs	Note in the genome	Signal peptide and cutting site	Signal sequence in details
GlmU	bifunctional UDP-N-acetylglucosamine pyrophosphorylase/glucosamine-1-phosphate N-acetyltransferase GlmU	MDNRDNGGQYM DKRFAV**VLA**|**A**G Q	1 ATGGATAATAGGGATAATGGAGGCCAATACATGGATAAGCGGTTTGCAGTTGTGTTAGCA 1 M D N R D N G G Q Y M D K R F A V V L A 70 80 90 100 110 120 61 GCTGGTCAAGGAACAAGAATGAAATCAAAGCTATATAAAGTTCTTCATCCTGTTTGCGGA 21 A G Q G T R M K S K L Y K V L H P V C G
YxaJ	putative ORF	M**K**TN**R**VSVMIWT LISAFLFCSMIVA ASLSPLAHSG**PH A**|NE	1 ATGAAAACCAATAGAGTAAGTGTAATGATCTGGACTTTAATCTCCGCTTTTCTGTTTTGT 1 M K T N R V S V M I W T L I S A F L F C 70 80 90 100 110 120 61 TCTATGATAGTCGCAGCTTCACTTTCTCCGCTCGCTCATTCAGGCCCTCATGCTAATGAG 21 S M I V A A S L S P L A H S G P H A N E 130 140 150 160 170 180 121 TTTGGCACTTTGGGGATGTGGGCGGCAATCGGAACCGTTTTGCTCTTTTATATGCTGCCC 41 F G T L G M W A A I G T V L L F Y M L P
YbxG	amino acid permease	MANKEL**KR**GLG ARHIQMIALGGTI GVGLFMGSSS**TI *K*|**WT	1 GTGGCGAACAAAGAATTGAAGAGAGGCCTGGGCGCGCGCCACATCCAAATGATTGCGCTT 1 V A N K E L K R G L G A R H I Q M I A L 70 80 90 100 110 120 61 GGCGGCACTATCGGCGTCGGTTTATTTATGGGATCTTCCAGCACGATAAAGTGGACAGGA 21 G G T I G V G L F M G S S S T I K W T G 130 140 150 160 170 180 121 CCTTCCGTCCTGCTTGCTTATGCCATTTGCGGAATTTTTATCTTTTTTATTATGCGTGCA 41 P S V L L A Y A I C G I F I F F I M R A
YbgF	putative amino acid permease YbgF	MEQSNTN**R**QNF **QRK**MQTRHLIML SLGGVIGTGLFLS SGYTI**SQA**|**G**P	1 ATGGAACAGTCAAACACCAATCGCCAGAACTTTCAAAGAAAAATGCAAACGAGACACCTT 1 M E Q S N T N R Q N F Q R K M Q T R H L 70 80 90 100 110 120 61 ATCATGCTTTCCTTAGGAGGCGTTATCGGCACGGGCCTTTTCTTAAGTTCCGGTTATACG 21 I M L S L G G V I G T G L F L S S G Y T 130 140 150 160 170 180 121 ATCTCACAGGCTGGTCCTGCCGGAACGATTCTAGCCTACTTGGCCGGGGCTCTGATCGTC 41 I S Q A G P A G T I L A Y L A G A L I V
PhoD	phosphodiesterase/alkaline phosphatase	M**KK**LSEESL**K**DN TFD**RRR**FIQGAG KIAGLSLGLAIAQ SMGAME**VNA**|**A**P	1 ATGAAAAAACTGAGCGAGGAAAGCCTCAAGGACAATACGTTTGACCGCCGCCGCTTTATT 1 M K K L S E E S L K D N T F D R R R F I 70 80 90 100 110 120 61 CAAGGGGCCGGCAAAATAGCCGGGCTTTCGCTCGGACTTGCGATCGCGCAATCGATGGGG 21 Q G A G K I A G L S L G L A I A Q S M G 130 140 150 160 170 180 121 GCAATGGAAGTCAATGCAGCACCGAGGTTCTCCGAATATCCGTTTACACTTGGCGTTGCA 41 A M E V N A A P R F S E Y P F T L G V A 190 200 210 220 230 240 181 TCGGGAGATCCGCTTTCTGACAGCGTCGTATTGTGGACAAGGCTGGCGCCCGATCCGCTA 61 S G D P L S D S V V L W T R L A P D P L
YcgH	putative amino acid transporter YcgH	M**KR**QA**KK**GDL**K** WWQLSMIGVGC TIGTGFFLGSSI**AI *K*|**KS	1 ATGAAACGACAAGCGAAAAAAGGAGATTTAAAATGGTGGCAGCTGTCCATGATCGGGGTC 1 M K R Q A K K G D L K W W Q L S M I G V 70 80 90 100 110 120 61 GGCTGCACAATTGGAACGGGGTTTTCCTCGGCTCAAGCATCGCGATTAAGAAAAGCGGC 21 G C T I G T G F F L G S S I A I K K S G 130 140 150 160 170 180 121 TTTTCCGTATTAGCCGCCTTTCTTCTTGCTGCCCTTGGCACTTTCTTGTTTTTCAGCAG 41 F S V L A A F L L A A L G T F L V F Q Q
YdbS	cytosolic protein YdbS	M**R**SEP**K**NQIS**R**D GV**K**VW**R**ITACITS FVLMLAAAG**LIA**| **A**G	1 TTGAGAAGCGAGCCGAAAAATCAAATCAGCCGTGATGGCGTTAAAGTCTGGAGAATCACG 1 L R S E P K N Q I S R D G V K V W R I T 70 80 90 100 110 120 61 GCTTGTATCACATCATTCGTTTTAATGCTGGCTGCAGCAGGCCTGATTGCCGCCGGGGTG 21 A C I T S F V L M L A A A G L I A A G V 130 140 150 160 170 180 121 ATATTTAAGTGGCCGGTATGGATCGGCATTTTGTCAGCGGCCGTTTGGCTGGGGATTTCA 41 I F K W P V W I G I L S A A V W L G I S

Table 4 (*continued*)

YhdH	putative sodium-dependent transporter YhdH	MSD**RR**MTA**K**WA S**K**LGFVLAAAGS AIGLGAIWKFP**YV** **A**I**G**	1 1 61 21 121 41	ATGAGCGACCGCAGAATGACAGCAAAGTGGGCATCCAAACTTGGATTTGTTCTCGCCGCC M S D R R M T A K W A S K L G F V L A A GCGGGGTCCGCCATCGGTCTGGGAGCGATTTGGAAGTTTCCCTATGTGGCGGGAACGAGC A G S A I G L G A I W K F P Y V A G T S GGGGGAGGCGCATTTTTCCTTGTGTTTGTCCTATTTACGATACTGCTGGGATACCCCCTG G G G A F F L V F V L F T I L L G Y P L
YhjN	putative ammonia monooxygenase YhjN	MILISSFGGFLLS LTGMTIGWMVGT MTAAACLSLFRP SP**L*K*A**I**A**V	1 1 61 21 121 41	TTGATATTGATCAGCAGTTTTGGGGGATTTCTTCTCTCATTGACCGGCATGACAATCGGC L I L I S S F G G F L L S L T G M T I G TGGATGGTCGGAACAATGACCGCCGCCGCTTGTCTGTCGCTGTTTCGCCCGTCACCGTTG W M V G T M T A A A C L S L F R P S P L AAAGCGGCGGTCAGACAAAACGGCATCCACCATGGATGGCTTCAGTTCGGCCAAATGCTA K A A V R Q N G I H H G W L Q F G Q M L
AbnA	arabinan endo-1,5-alpha-L-arabinosidase AbnA	ML**K**TS**K**FE**RR**TT VAKTIISGFILFFL LI**FSA**I**A**EP	1 1 61 21 121 41	ATGTTAAAGACATCGAAATTTGAAAGGAGAACAACTGTGGCTAAGACTATCATTTCTGGT M L K T S K F E R R T T V A K T I I S G TTTATTTTATTTTTTCTGCTTATTTTCTCGGCGGCTGAACCAACATCAGCGGCTTTTTGG F I L F F L L I F S A A E P T S A A F W GATACAAAAGGAGACAACATTATTCATGATCCTTCCATGATTAAAGAAGGAAATACGTGG D T K G D N I I H D P S M I K E G N T W
YesL	putative membrane protein YesL	MIHTLANGFY**R**F CEWVM**R**LAYLNL LWIGFTLAGAVIF GLAP**ATA**I**A**M	1 1 61 21 121 41	ATGATTCACACGTTGGCAAATGGATTTTACCGCTTTTGCGAGTGGGTGATGCGGCTTGCC M I H T L A N G F Y R F C E W V M R L A TATTTGAATCTGCTGTGGATCGGCTTTACGCTGGCGGGAGCGGTCATCTTCGGTTTAGCG Y L N L L W I G F T L A G A V I F G L A CCGGCGACCGCCGCGATGTTCGCTGTGACTAGACAGTGGACGCTGGGAAAAACGGATGTC P A T A A M F A V T R Q W T L G K T D V

volume of 50 ml. When fermenting in a 25-l bioreactor (Bioflow110; New Brunswick Scientific Co., Inc., Edison, NJ), the recombinant strain was incubated at 42°C, 1 vvm aeriation and 200–700 rpm agitation with the initial working volume of 10 l. When necessary, a total of 800 g of lactose in 300 g/l of lactose solution was fed.

2.5. PGA activity assay

PGA activity was assayed according to reference [2] using Cbz-Gln-Gly (Peptide Laboratory, Shanghai, China) as a substrate at pH 6.5 and 37°C. One unit of PGA is defined as the amount of enzyme that releases 1 μmol of ammonium per minute under the assay conditions specified.

2.6. Database mining of B. licheniformis Tat signal peptides

The signal peptide prediction of twin-arginine signal peptides in *B. licheniformis* ATCC 14580 genome was made using TatP 1.0 (http://www.cbs.dtu.dk/services/TatP/).

3. Result and discussion

3.1. Analysis of B. licheniformis Tat signal peptides

Based on the consensus sequence of Tat signal peptides, all putative twin-arginine signal peptides in *B. licheniformis* ATCC 14580 genome were predicted using TatP 1.0 server according to the presence of an RR(FGAVML)(LITMVF) motif [14]. A total of 4156 putative ORFs in the genome sequence were analyzed. Of which, 58 ORFs containing putative twin-arginine signal peptides were finally predicted. Their hydrophobic core (H-domain) had an average length of 18 amino acid residues. Their SPases I recognition sequences consisted of small aliphatic residues at positions -3 and -1. The preferred alanine and lysine residues were at the position -1 and the residue, such as alanine, lysine, serine, valine, arginine or leucine accommodated the position -3 (Table 3). The most frequent residue was alanine accounting at positions -3 and -1 for 39.7% and 83.8%, respectively (Table 3). Of total 58 putative Tat signal peptides in *B. licheniformis,* 11 putative Tat signal peptides were randomly selected for further study and their sequences are summarized in Table 4.

3.2. Tat-dependent secretion of PGA mediated by Tat signal peptides of B. licheniformis

To illustrate the possibility of the Tat signal peptides from *B. licheniformis* for mediating PGA secretion, the nucleotide sequences encoding for 11 selected twin-arginine signal peptides were recovered from *B. licheniformis* ATCC 14580 genome by PCR amplification. The PCR products were purified and digested with *Bam*HI, and then cloned into the plasmid pHY-WZX to replace the AmyL signal peptide [13]. A total of 11 expression plasmids, nominated as pHY-TAT$_{1\sim11}$, were constructed, which were further confirmed by PCR amplification, restriction pattern and nucleotide sequencing (Fig. 1).

To express the pro-PGA, the chemically synthesized pro-PGA coding sequence was recovered, digested with *Bam*HI, and cloned into each of pHY-TAT plasmids digested with *Bam*HI and *Sma*I, yielding recombinant plasmid pHY-PGAs. The transformants were obtained by transforming the recombinant plasmid pHY-PGAs into *B. subtilis* WB600. The fermentation tests were carried out and the PGA activities in the supernatant were examined. The activities are summarized in Table 5. The twin-arginine signal peptides of GlmU, YhdH and YxaJ were able to mediate the PGA secretion in *B. subtilis*. Of which, GlmU twin-arginine signal peptide mediated the highest PGA secretion and YhdH and YxaJ twin-arginine signal peptides mediated very weak but detectable PGA secretion (Table 5). GlmU in *B. licheniformis* is a

Table 5
B. licheniformis twin-arginine signal peptides mediated PGA secretion in *B. subtilis*.

Tat signal peptide	PGA activity (mU/ml)
GlmU	160 ± 25
YxaJ	N.D.[a]
YbxG	57 ± 13
YbgF	N.D.
PhoD	N.D.
YcgH	N.D.
YdbS	N.D.
YhdH	24 ± 15
YhjN	N.D.
AbnA	N.D.
YesL	N.D.
AmyL	N.D.

[a] N.D.: Not detectable.

putative (yet its function not identified) bifunctional UDP-N-acetylglucosamine pyrophosphorylase/glucosamine-1-phosphate N-acetyltransferase for the synthesis of UDP-N-acetylglucosamine [15].

Several previous reports indicated that the PGA might fold too rapidly to be treated by the heterologous Sec machinery [8,16]. Different from the Sec pathway, the Tat pathway tends to translocate its substrates in a folded conformation [17,18,19]. Using Tat signal peptides from *E. coli* TorA or *A. globiforimis* IMD, PGA was successfully secreted with the Tat pathway in *C. glutamicum* [4]. In this report, we found that three twin-arginine signal peptides from *B. licheniformis* were able to indicate the production of PGA in *B. subtilis* in active form and the different secretion efficiencies were also observed (Table 5). Interestingly, PGA in *B. subtilis* WB600 was produced in active form without additional processing, for example, being converted to an active form by removing the propeptide with a subtilisin-like serine protease SAM-P45 [8]. These results suggested that an unknown peptidase accurately digested pro-PGA to active one in *B. subtilis* WB600 although its six extracellular proteases (neutral protease A, subtilisin, extracellular protease, metalloprotease, bacillopeptidase F, and neutral protease B) were deleted [11]. *B. subtilis* WB600 kept 0.32% of the wild-type extracellular protease activity and no residual protease activity could be detected when it was cultured in the presence of 2 mM phenylmethylsulfonyl fluoride [11].

3.3. Modification of N-terminal of GlmU twin-arginine signal peptide improved PGA secretion

The positive charge of the N-region is generally considered to be essential for recognition by translocase to initiate protein translocation and to interact with the negatively charged lipids at the cell membrane as well although its importance is uncertain [20]. The third residue, aspartic acid (N) of GlmU twin-arginine signal peptide, was replaced with lysine (K) or arginine (R) by site-directed mutagenesis. Two mutants, GlmU-K and GlmU-R, were obtained (Fig. 2A). Their effects of amino acid alterations on the secretory performance of PGA were evaluated. The change of the N-regions amino acid residues from N to K (GlmU-K) or to R (GlmU-R) resulted in a different level of PGA secretion in *B. subtilis* WB600. GlmU-R mediated the improved secretion of PGA with about 40% increase (230 mU/ml). GlmU-K did, however, reduced the secretion of PGA (Fig. 2B).

It was found that the N-region's positive charge density in alkaline phosphatase signal peptide increased the secretory efficiency of *E. coli* alkaline phosphatase [21]. The mutant GlmU-R developed in this study was to increase the N-region's net charge by the introduction of a positive charge in the form of arginine residue, which might also make it more susceptible by a signal peptide peptidase [22].

3.4. Over-expression of PGA in B. licheniformis

To examine the possibility of PGA production in *B. licheniformis*, the recombinant plasmid pHY-PGAr was transformed into *B. licheniformis* CBBD302, an industrial enzyme expression host [10]. The yield of PGA in *B. licheniformis* was determined in a 25-l fermentor (Fig. 3). With no surprise, PGA was produced and secreted in its active form in *B. licheniformis*. The maximal yield was 6.8 U/ml in the supernatant. To our knowledge, this is the highest yield ever reported.

4. Conclusion

In conclusion, PGA can be efficiently produced and secreted in active form via Tat pathway of *B. licheniformis*, an efficient alternative for the industrial-scale production of PGA.

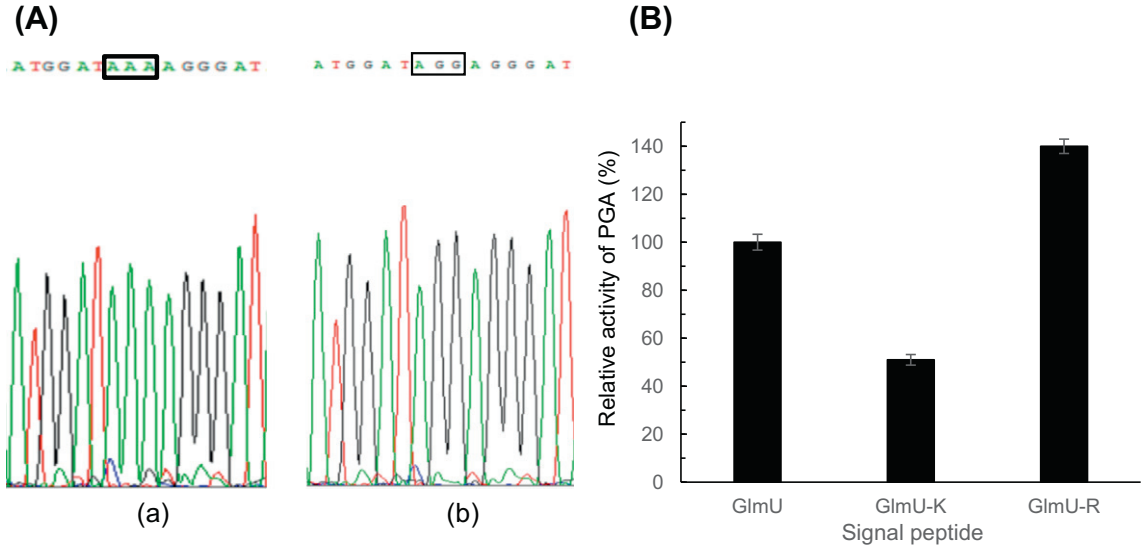

Fig. 2. Effects of mutant GlmU signal peptide on the secretion of PGA. (A)The sequencing analysis of the site-directed mutants of GlmU, GlmU-K (a) and GlmU-R (b). (B) Secretory activity of PGA mediated by GlmU mutants. The activity mediated by wild-type GlmU was taken as 100%.

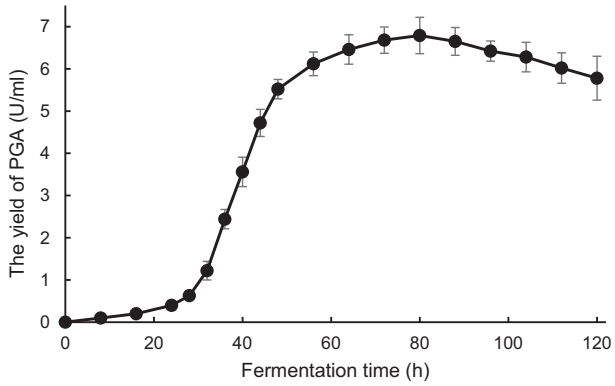

Fig. 3. The time-cause of PGA production in *B. licheniformis*. The production of PGA was taken place in a 25-l bioreactor with initial working volume of 10 l. LB complemented with 40 g/l of lactose was used as medium. During the fermentation about 267 ml of 300 g/l lactose (total 800 g) was fed.

Financial support

This study was funded by National Natural Science Foundation of China (31601407) and in part by the Intergovernmental International Scientific and Technological Innovation Cooperation Program, MOST, China (2018YFE0100400).

Conflict of interest

The author(s) declare that they have no competing interests.

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