Construction of Chromosomally Located T7 Expression System for Production of Heterologous Secreted Proteins in *Bacillus subtilis*

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Bacillus subtilis is most commonly employed for secretion of recombinant proteins. To circumvent the problems caused by using plasmids, the T7 expression system known for its high efficiency was rebuilt in *B. subtilis*. Accordingly, a markerless and replicon-free method was developed for genomic insertion of DNAs. By the act of homologous recombination via the guide DNA, a suicidal vector carrying the gene of interest was integrated into genomic loci of bacteria. Removal of the inserted selection marker and replicon flanked by FRT sites was mediated by the FLP recombinase. By using the mentioned system, *B. subtilis* strain PT5 was constructed to harbor a genomic copy of the spac promoter-regulated *T7 gene 1* located at *wprA* (encoding the cell wall-associated protease). Similarly, the T7 promoter-driven nattokinase or endoglucanase E1 of *Thermomonospora fusca* genes were also integrated into *mpr* (encoding an extracellular protease) of strain PT5. Consequently, the integrant PT5/Mmp-T7N or PT5/MT1-E1 resulted in a "clean" producer strain deprived of six proteases. After 24 h, the strain receiving induction was able to secret nattokinase and endoglucanase E1 with the volumetric activity reaching 10860 CU/mL and 8.4 U/mL, respectively. This result clearly indicates the great promise of the proposed approach for high secretion of recombinant proteins in *B. subtilis*.

KEYWORDS: *Bacillus subtilis*; T7; FLP recombinase; integration

INTRODUCTION

Bacillus subtilis is a "generally recognized as safe" (GRAS) organism and, particularly, highly competent for protein secretion (*1*). With these traits, this species has become a superior surrogate cell of choice for secreted production of proteins, thereby facilitating the downstream processing of proteins. For the engineering purpose, there are needs to obtain huge amounts of proteins of interest. This would strongly rely on the producer cells being capable of expressing the target genes carried on plasmids. Unfortunately, constructed vectors employed in *B. subtilis* were notorious for their instability (*2*). Two events, known as segregational and structural instability of plasmids, have been well described (*3, 4*). To tackle this problem, plasmid constructs replicating in a θ mode were developed to express full structural stability with controlled expression (*5−7*). Recently, we have also constructed a new shuttle vector derived from plasmid pUB110 of *Staphylococcus aureus* and plasmid R6K of *Escherichia coli* (*6*). As illustrated, this plasmid exhibited high stability to achieve high production of secreted nattokinase (*6, 8*) and *Renilla luciferase* (*9*).

As first described in *E. coli*, the T7 expression system is known for its high efficiency and specificity in expression of target genes under the control of the T7 promoter (*10*). Initiation of the gene transcript from the T7 promoter requires the function of T7 RNA polymerase (encoded by *T7 gene 1*). As a consequence, it permits the exclusive expression of the T7 promoter-driven genes without competing for the host RNA polymerase (*11*). For most common practices, an *E. coli* strain bearing a chromosomal copy of *T7 gene 1*, controlled by the *lacUV5* promoter, is used for high-level expression of genes on a plasmid upon induction by β-ᴅ-thiogalactopyranoside (IPTG). Therefore, by regulation of *T7 gene 1* with distinct promoters, recombinant proteins could be produced in response to a variety of inducers (*12, 13*). Owing to its prominent merits and high flexibility, this system has been applied to other host organisms, including mammalian cells (*14, 15*) and yeast (*16, 17*).

The approach of maintaining a high copy of genes with plasmids is usually reflected by an enhanced level of proteins produced. However, it closely accompanies a problem of plasmid burden, which leads to the physiological perturbation of host cells, consequently leading to segregational loss or internal rearrangements of plasmids and greatly discouraging protein yield (*18*). To circumvent the use of plasmids, a strong expression

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system is required for a high expression level of proteins when the encoding genes are otherwise placed in the host genome. Therefore, in this study we attempted to rebuild the T7 expression system in *B. subtilis*. For genomic insertion, the gene of interest that should be inserted was cloned into a suicidal vector, which contains an endogenous DNA and the FRT site-flanked DNA backbone consisting of an antibiotic-resistant determinant along with the *E. coli* plasmid origin. Guided by the endogenous DNA, the vector DNA was directed to integrate into the destined loci by homologous recombination. With this approach, a *B. subtilis* strain was constructed to harbor a genomic copy of the *T7 gene 1* under the control of the spac promoter. In a similar manner, the T7 promoter-regulated nattokinase (*19*) or endoglucanase E1 of *Thermomonospora fusca* (*20*) was integrated into the host genome. By the action of FLP recombinase (*21, 22*), the selection marker and the plasmid origin inserted in the host genome were then eliminated. Consequently, a producer strain free of replicons and selection markers was produced and shown to secrete a large amount of nattokinase and endoglucanase E1. This result clearly indicates the great promise of the proposed approach for high secretion of recombinant proteins in *B. subtilis*.

MATERIALS AND METHODS

Bacterial Strains and Culture Condition. Manipulation of DNAs was routinely conducted in *E. coli* strains BW25142 (*lacI^q rrnB3 ΔlacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568 ΔphoBR580 rph-1 galU95 Δ endA9 uid*A(*ΔMlu*I)::*pir-116 recA1*) (*23*) and DH5α (*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15*). *B. subtilis* strain DB428 (*trpC2 nprE aprE EPR bpf*) obtained from Bacillus Genetic Stock Center (BGSC) was engineered to equip the T7 expression system for secreted production of heterologous proteins. To perform the experiments, recombinant strains were grown in shake flasks containing Luria–Bertani (*24*) medium while their growth was monitored turbidimetrically at 600 nm (OD_{600}). After overnight growth, the cultures were inoculated to get an initial cell density of 0.1 at OD_{600}. The cells was then maintained in an orbital shaker set at 200 rpm and 37 °C. Ampicillin (100 μg/mL for *E. coli*), kanamycin (50 μg/mL for *E. coli* and 10 μg/mL for *B. subtilis*), and erythromycin (10 μg/mL for *B. subtilis*) were used to select *E. coli* or *B. subtilis* strains harboring plasmids.

Plasmid Construction. Plasmids and primers used in this study are listed in **Table 1**. The integration vector pMFRT-GFP containing two FRT sites was generated using primers PT003–PT006 with pMutin-GFP (*25*) as the template by Polymerase Chain Reaction (PCR). The *T7 gene 1* was obtained from strain *E. coli* BL21(DE3) by PCR using primers PT007–PT008. Meanwhile, the truncated *mpr* (*mpr′*) and *wprA* (*wprA′*) genes were amplified from *B. subtilis* DB428 based on two pairs of primers, PT009–PT010 and PT045–PT046, respectively. The PCR DNAs containing *mpr′* and *wprA′* were cleaved with *Bgl*II–*Eco*RI and *Xba*I–*Eco*RI, respectively. Subsequent ligation of digested DNA fragments into pMFRT-GFP resulted in pMmp-GFP and pMwp-GFP constructs. Digested by *Hind*III and *Sal*I, the *T7 gene 1* was spliced into pMwp-GFP to generate pMwp-T7gene1 (**Figure 1A**).

The *E. coli*/*B. subtilis* shuttle vector, pEKA, was constructed as follows. By PCR, the pE194 origin, R6K origin, and spac promoter with *lacI* were generated from pE194 (BGSC), pUKVI (*6*), and pULI7 (*26*) using the corresponding sets of primers EK001–EK002, EK003–EK004, and EK005–EK006, respectively. Three PCR DNAs were spliced together to give pEKA. Moreover, the FLP recombinase gene was synthesized from pCP20 (*27*) with primers PT105–PT106 and incorporated into pEKA pretreated with *Xba*I and *Pst*I to produce pEKA-FLP (**Figure 1C**).

To clone the *aprN* gene encoding nattokinase, oligomers PT011 and PT012 were used to prime the chromosome of strain *Bacillus natto* (SynbioTech Co., Taiwan). After cleavage with *Xba*I and *Sal*I, the PCR product was spliced into pET-20b(+) to give plasmid pET-NAT. Subsequently, the DNAs containing *aprN* fused with T7 promoter and T7 terminator were obtained from pET-NAT with primers PT015–PT016 and PT016–PT099 using PCR. By treatment with the *Eco*RI–*Bam*HI and *Eco*RI–*Nar*I cuts, the DNAs incorporated into pUKVI and pMmp-GFP to generate pUKT-NAT and pMmp-T7N (**Figure 1B,D**), respectively.

Moreover, integration vector pMT1 carrying the T7 promoter with the *aprN* signal peptide was created by amplifying the structural gene of *aprN* from pMmp-T7N using PCR with primers MT001–MT002. The endoglucanase E1 without the leader peptide was then synthesized from *Thermomonospora fusca* by PCR with oligomers PT051–PT034. The resulting gene was digested with *Xho*I and *Bam*HI and subcloned into pMT1 to give pMT1-E1 (**Figure 1E**).

Construction of Replicon- and Marker-Free Producer Strains. All plasmids were constructed in *E. coli* and used to transform into *B. subtilis*. As described previously (*28*), the integrants and plasmid-bearing *B. subtilis* strains were constructed by electroporation and selected against kanamycin or erythromycin. In brief, 0.1–1 μg of plasmid DNAs was added into 40 μL of the chilled competent cells. After transfer into a 1 mm ice-cold electroporation cuvette for 1.5 min, the mixture was exposed to a single pulse using a BTX BCM 630 electroporator. The operation condition was set for a peak discharge of 12.5 kV/cm, 25 μF, and 200 ohm. Subsequently, 1 mL of recovery medium (LB plus 0.5 M sorbitol and 0.38 M mannitol) was added into the cuvette immediately post electrical discharge. The cells were plated on LB agar plates containing the indicated antibiotics following incubation at 37 °C for 3 h.

To eliminate the region containing the selective markers and the *E. coli* replicon origin, integrants bearing the inserted DNA were transformed with plasmid pEKA-FLP expressing FLP recombinase. The resulting cells were grown on LB medium at 37 °C and induced by adding 0.5 mM IPTG upon OD_{600} reaching 0.1. After overnight cultivation, the integrants were spread on nonselective LB agar plates at 39 °C. The cell-forming units were then examined for their susceptibility to antibiotics.

Analytical Methods. Cells of *B. subtilis* were harvested by centrifugation for the determination of enzyme activity and for the analysis of protein production by 8% (w/v) SDS-PAGE as previously described (*13*).

To determine the nattokinase activity (in *B. subtilis*), 0.1 mL of culture broth was added into 1 mL of a solution containing 0.1 M sodium phosphate buffer (pH 7.5) and 5% (w/v) casein. The reaction was carried out at 37 °C for 3 min and quenched by adding 2 N HCl (0.1 mL). After centrifugation at 4 °C, the supernatant was removed and measured at 275 nm. One unit of caseinolytic activity was defined as the enzymatic release of 1 μmol of tyrosine equiv/min.

Cellulase activity toward carboxyl methyl cellulose (CMC) was measured according to the previous method (*29*). The reaction mixture was composed of 0.5 mL of CMC (1%) in 50 mM sodium phosphate buffer (pH 7.0) and 0.5 mL of supernatant. After incubation at 55 °C for 15 min, the concentration of reducing sugars was determined using the dinitrosalicylic acid (DNS) method. One unit of enzyme activity corresponds to 1 μmol of glucose/min.

RESULTS

Construction of the *B. subtilis* Strain Carrying a Genomic Copy of *T7 gene 1*. The general procedure for administration of genomic insertion of DNAs is outlined in **Figure 2A**. This approach allows insertion of foreign genes into *B. subtilis* chromosome and later elimination of most of the integrated plasmid DNA comprising the selective marker and the plasmid replication origin of *E. coli*. Accordingly, integration plasmid pMwp-T7gene1 (**Figure 1A**) with the passenger gene, consisting of lacI and *T7 gene 1* fused to the spac promoter, was transformed into *B. subtilis* strain DB428. Subsequently, integrants were screened for exhibiting resistance to erythromycin, and the integration event was verified using in situ PCR. As shown in **Figure 2B**, this revealed the inserted DNA fragment in the *wprA* gene (encoding the cell wall-associated protease) with the expected size (lane 1). The resulting strain was designated DB428 (T7g).

As illustrated in **Figure 2A**, the inserted plasmid DNA flanked by the FRT site can be removed by FLP recombinase. Therefore, plasmid pEKA-FLP was constructed to express FLP under control of the IPTG-inducible spac promoter (**Figure 1C**). After transformation of plasmid pEKA-FLP, strain DB428(T7g) was grown and induced for production of FLP recombinase by IPTG. The resulting cells were then examined for their sensitivity to

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Table 1. Strains, Plasmids, and Primers Used in This Study[a]

	relevant characteristics	source
strain		
E. coli		
DH5α	*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15*	lab collection
BW25142	*lacI*^{q}*rrnB3 ¢lacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568 ¢phoBR580 rph-1 galU95 ¢endA9 uidA*(¢*MluI*)::*pir-116 recA1*	lab collection
B. subtilis		
DB428	*trpC2 nprE aprE EPR bpf*	BGSC
DB428(T7g)	DB428, Δ*wpr::T7gene 1-erm*	this study
PT5	DB428, Δ*wpr::T7gene 1*	this study
PT5(Mmp-T7N)	PT5, Δ*mpr*:: P$_{T7}$ /*aprN*	this study
PT5(MT1-E1)	PT5, Δ*mpr*:: P$_{T7}$ /E1	this study
plasmid		
pET-20b(+)	*E. coli* expression vector	
pET-NAT	*E. coli* expression vector contains nattokinase driven by P$_{T7}$	this study
pMutin-GFP	integration vector with GFP	BGSC
pMmp-GFP	pMUTIN-GFP with *mpr'*	this study
pMwp-GFP	pMUTIN-GFP with *wprA'*	this study
pUKVI	*B. subtilis*/*E. coli* shuttle vector	
pCP20	helper plasmid expressing FLP	
pMFRT-GFP	pMUTIN-GFP with FRT sites	this study
pMwp-T7gene1	pMFRT-GFP with T7 gene 1 and *wprA'*	this study
pMmp-T7N	integration vector contains nattokinase driven by P$_{T7}$ and *mpr'*	this study
pMT1	integration vector contains P$_{T7}$ and *aprN* SP	this study
pMT1-E1	pMT1 with *T. fusca* E1	this study
pE194	*B. subtilis* template vector	
pEKA	*B. subtilis*/*E. coli* shuttle vector	
pULI7	*B. subtilis* template vector	
pEKA-FLP	*B. subtilis*/*E. coli* shuttle vector with FLP driven by P$_{spac}$	this study
pUKT-NAT	*B. subtilis*/*E. coli* shuttle vector with nattokinase driven by P$_{T7}$	this study
primer		
PT003	AGGAACTTCAGATCTTCGCGACTGCAGGAATTCTACACAGCCCAGTC	
PT004	ATTCTCTAGAAAGTATAGGAACTTCCATAGAATTATTTCCTCCCGT	
PT005	AGAATAGGAACTTCGGAATAGGATACCGCATCAGGCGCTC	
PT006	CTAGAAAGTATAGGAACTTCCGCTCACTGCCCGCTTTC	
PT007	CAATTAAGCTTAAGGAGAGAAGTAACCATGAACACGATTAACATCGCT (*Hind*III)	
PT008	TGGCGGTCGACTTACGCGAACGCGAAGTC (*Sal*I)	
PT009	GCAGCAGCGAGATCTTTTGGCGTAC (*Bgl*II)	
PT010	CCGCTTCTGCAGCCGTACGTATC (*Pst*I)	
PT011	GTCTACTCTAGATTTTTTAAAAGGAG (*Xba*I)	
PT012	CTAGAGTCGACTTATTGTGCAGCTGCT (*Sal*I)	
PT015	TTCCTCGGATCCGCAAAAAACCCCTCAAGAC (*Bam*HI)	
PT016	GGCCAGGAATTCACGCTGCCCGAGATCTC (*Eco*RI)	
PT034	ACATCCGGATCCAGGGCGAGGTTC (*Bam*HI)	
PT045	CTATCAAGATCTAGAGACATTAAAGCCGTTG (*Xba*I)	
PT046	AAACTTGAATTCATCACCTTGGCTCCTTTG (*Eco*RI)	
PT051	CCGCCTCGAGAGACGAAGTCAACCAGATTC (*Xho*I)	
PT099	TCTAGAGGCGCCGCAAAAAACCCCTCAAG (*Nar*I)	
PT105	CAATTAAGCTTGGAGGGGTTCTAGAATGTCACAATTTGATATATTATGTAAAACAC (*Xba*I)	
PT106	TGCATGCCTGCAGACTTATATGCGTC (*Pst*I)	
EK001	GAAAGGCCTAGCGACAGCTATTAACTTTCGGT (*Stu*I)	
EK002	CCGTTTCTCGAGTTTTGCGCAGTCGGCTTAAACCAG (*Xho*I)	
EK003	GCAAAACTCGAGAAACGGGTTGACATGTTGAAG (*Xho*I)	
EK004	TAATGTGAGCTCCGCGATAGGAACTAAGGAG (*Sac*I)	
EK005	ATCGCGGGAGCTCACATTAATTGCGTTGCGCTCACTG (*Sac*I)	
EK006	GTCGCTAGGCCTTTCGATCGAATTCTACACAGCCCAGTC (*Stu*I)	
MT001	TTTTGCCTCGAGGGATCCCTGCAGGCATGCAAGCTAGCCACCACCACCACCACCACTGAG (*Xho*I)	
MT002	TCCATCCTCGAGGCAGCCTGCGCAGACATGTTG (*Xho*I)	

[a] Abbreviations: erm, erythromycin resistant determinant; E1, *T. fusca* E1 gene; Pspac, spac promoter; P7, T7 promoter; SP, signal peptide.

erythromycin. Furthermore, the occurrence of the DNA deletion event in the cells was confirmed by PCR. Among them, one deleted integrant was characterized and designated PT5 (lane 2, **Figure 2B**). Consequently, strain PT5 was deficient in five protease genes and carried a chromosomal copy of *T7 gene 1* under control of the spac promoter and *lacI*.

T7 RNA Polymerase-Driven Production of Nattokinase. As described, plasmid pUKT-NAT was constructed by fusion of

the nattokinase-encoded gene (*aprN*) with the T7 promoter (**Figure 1B**). The T7-based system was first characterized by overlaying strain PT5 carrying plasmid pUKT-NAT (PT5/ pUKT-NAT) on LB agar containing 1% skim milk. As a result, it gave rise to the formation of halos, indicating the active functionality of the T7 expression system for expressing nattokinase in *B. subtilis*. Moreover, strain PT5(Mmp-T7N) was constructed with the genomic insertion of the T7 promoter-driven
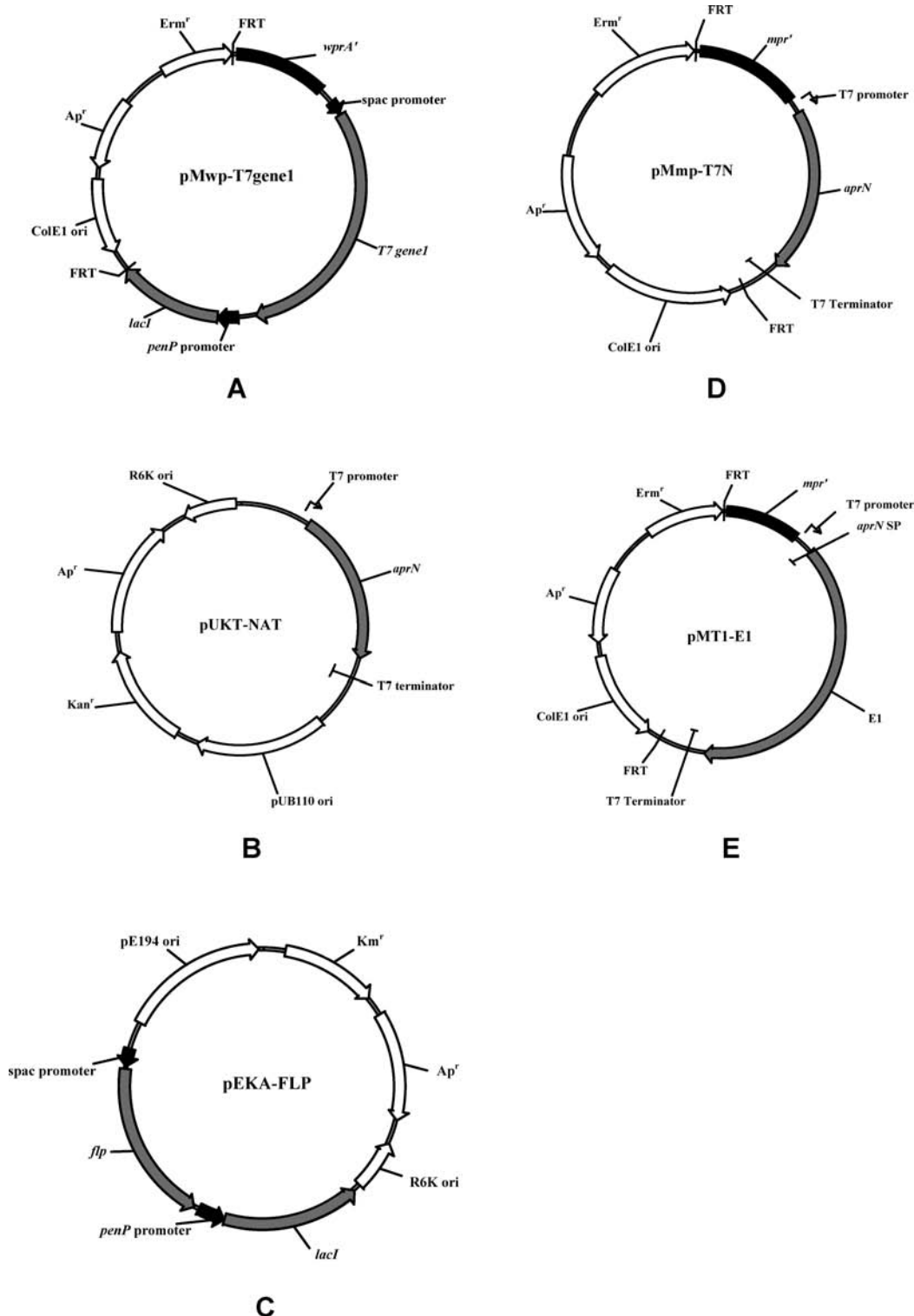
Figure 1. Physical maps of integration and shuttle vectors constructed in this study: integration vectors, pMwp-T7gene1, pMmp-T7N, and pMT1-E1, containing the ColE1 replication origin for propagation in *E. coli*; shuttle *E. coli*— *B. subtilis* vectors, pUKT-NAT and pEKA-FLP, carrying either R6K/pUB110 or R6K/pE194 replication origins. Abbreviations: Ap[r], ampicillin resistance determinant; E1, *T. fusca* endoglucanase E1 gene; Erm[r], erythromycin resistance determinant; *flp*, FLP recombinase gene; Kan[r], kanamycin resistance determinant; ori, replication origin; SP, signal peptide.

aprN into *mpr* (encoding an extracellular protease). This construct was obtained by transformation of plasmid pMmp-T7N (**Figure 1D**) into strain PT5 as described in the scheme outlined in **Figure 2A**. Afterward, the integration and deletion events in the cell were verified by PCR, and the strain's phenotype was examined on the agar plate containing skim milk (data not shown).

To further characterize, strains PT5/pUKT-NAT and PT5-(Mmp-T7N) were grown in shake flasks at 37 °C. As a result, nattokinase was secreted and accumulated in a time-dependent manner for both strains (**Figure 3A**). Upon induction by IPTG, strain PT5(Mmp-T7N) was able to secret 50% more nattokinase than strain PT5/pUKT-NAT after 24 h. However, for strain PT5/pUKT-NAT higher production of nattokinase was detected
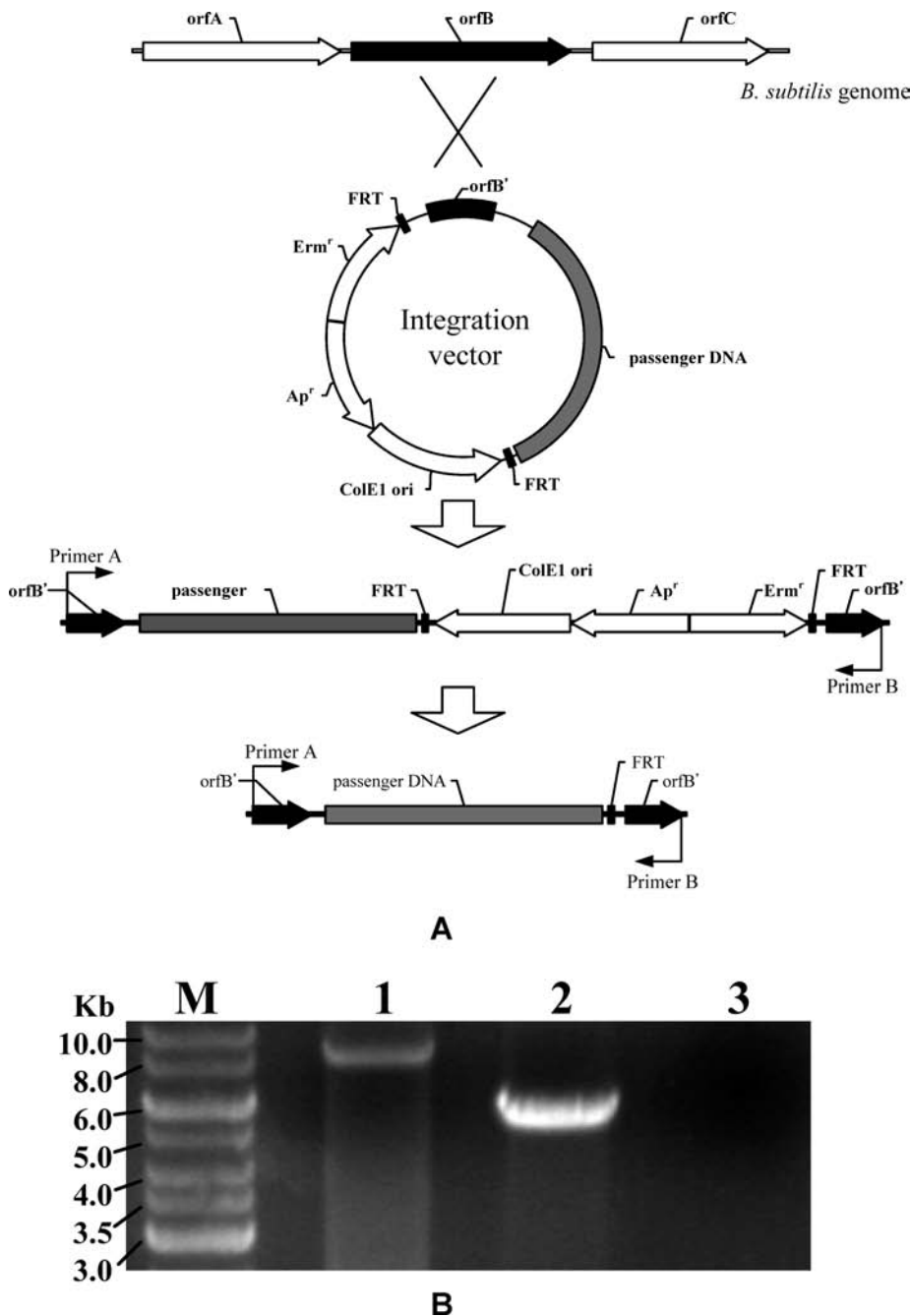
Figure 2. (**A**) Schematic illustration of the markerless and replicon-free method for insertion of DNAs into *B. subtilis* genome. The conditional-replication vector carrying the passenger gene is integrated into bacterial chromosome via the guide DNA (orfB′)-mediated homologous recombination. As a result, it endows the integrant gains with the ability to resist erythromycin. By the act of FLP recombinase provided from helper plasmid pEKA-FLP, the inserted replicon and selection marker flanked by FRT sites are eliminated, leading to the insertion of the passenger gene into the genomic locus (orfB). Upon shifting to 39 °C, the integrant is screened for both erythromycin and kanamycin sensitivity, indicating the loss of the selection marker and helper plasmid. The DNA insertion and deletion event was verified by in situ PCR using primers A and B. Abbreviations: orfA, open reading frame A; orfB, open reading frame B; orfB′, truncated orfB; orfC, open reading frame C. (**B**) Verification of the DNA insertion and deletion event by in situ PCR. With the primers PT045 and PT046 (corresponding to primers A and B as described in (**A**)), integrants were examined for genomic insertion of the passenger gene and the removal of the marker associated with replicon. Lanes: M, DNA marker; 1, inserted plasmid DNA (9.5 kb) in DB428(T7g); 2: inserted DNA consisting of *T7 gene 1* and *lacI* but without marker and replicon (6.5 kb) in PT5; 3, control strain DB428.

in uninduced than in induced culture (**Figure 3A,B**). Further analysis by SDS-PAGE showed that the unprocessed nattokinase in either the soluble or insoluble fraction was entrapped in the cell cytoplasm (**Figure 3C**). Overall, the result indicates that the secretion pathway of *B. subtilis* is likely saturated under these conditions.

Effect of the Inducer Concentration and Induction Time on Nattokinase Production. Protein production by the T7 system is

able to respond to two factors, concentration of inducer (dosage) and induction time. Therefore, their effects on nattokinase production were investigated using shake-flask cultures of strain PT5(Mmp-T7N). As indicated in **Figure 4A**, the nattokinase production was increased by increasing concentrations of IPTG and leveled off at 0.5 mM. However, protein production started to drop when IPTG exceeded 0.7 mM. In addition, the nattokinase production by the strain varied in response to various
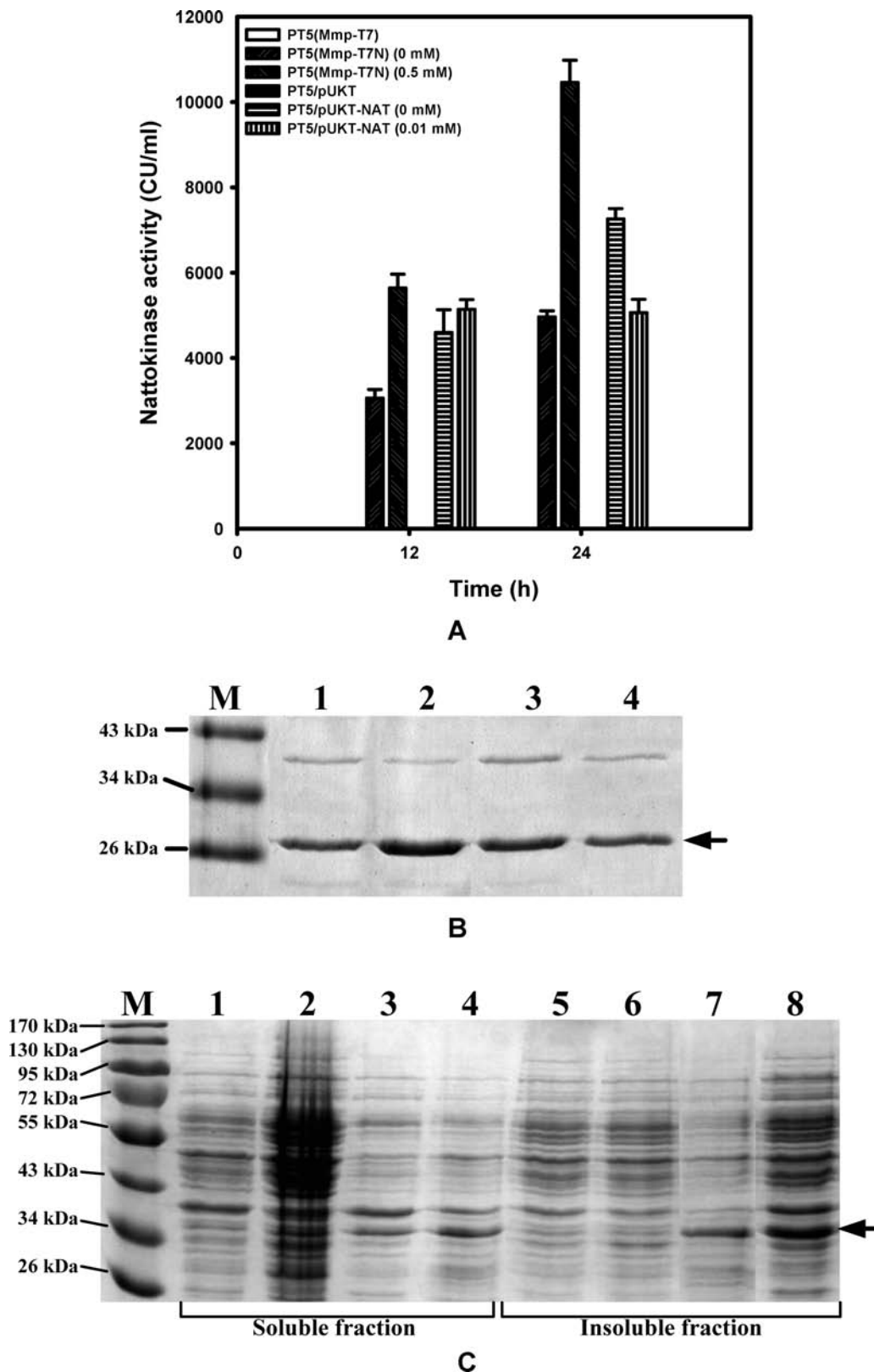
Figure 3. (**A**) Production of secreted nattokinase in *B. subtilis*. As described, the shake-flask cultures of producer strains were carried out while their production of secreted nattokinase at indicated time was examined by determination of enzyme activity using medium broth. The experiment was done in duplicate. (**B**) 8% (w/v) SDS-PAGE analysis of secreted nattokinase by *B. subtilis*. After cultivation for 24 h, the medium broth (12 μL) for growing each strain was analyzed by SDS-PAGE. Lanes: M, protein marker; 1, PT5(Mmp-T7N) without IPTG; 2, PT5(Mmp-T7N) with 0.5 mM IPTG; 3, PT5/pUKT-NAT without IPTG; 4, PT5/pUKT-NAT with 0.01 mM IPTG. (**C**) SDS-PAGE analysis of intracellular proteins in *B. subtilis*. After cultivation for 24 h, producer strains were harvested and disrupted by ultrasonication. Followed by centrifugation, the soluble and insoluble fractions containing 20 μg of proteins were resolved by SDS-PAGE. Lanes: M, protein marker; 1, soluble fraction of PT5(Mmp-T7N) without IPTG; 2, soluble fraction of PT5(Mmp-T7N) with 0.5 mM IPTG; 3, soluble fraction of PT5/pUKT-NAT without IPTG; 4, soluble fraction of PT5/pUKT-NAT with 0.01 mM IPTG; 5, insoluble fraction of PT5(Mmp-T7N) without IPTG; 6, insoluble fraction of PT5(Mmp-T7N) with 0.5 mM IPTG; 7, insoluble fraction of PT5/pUKT-NAT without IPTG; 8, insoluble fraction of PT5/pUKT-NAT with 0.01 mM IPTG. The position of nattokinase is indicated by an arrow.

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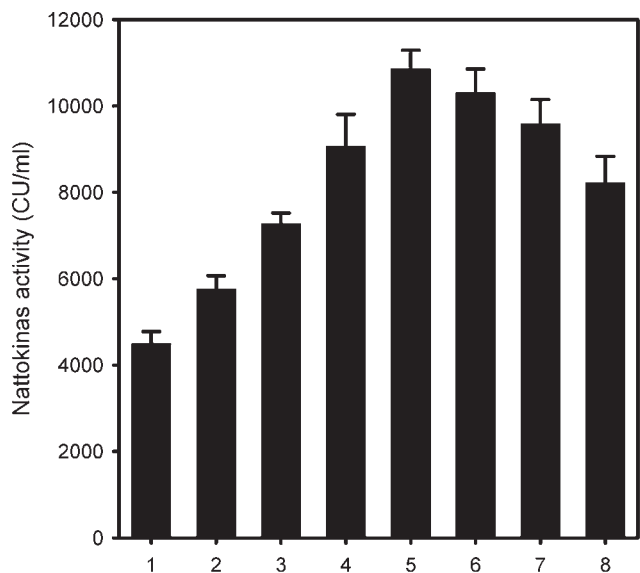
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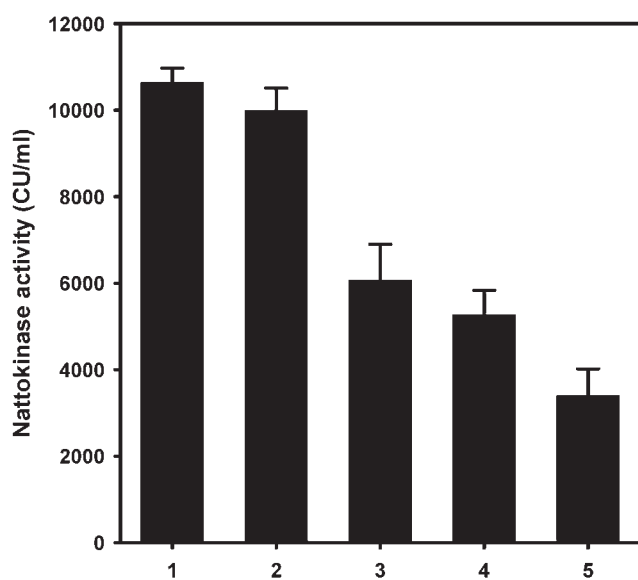
B

Figure 4. (**A**) Response of nattokinase production to various IPTG concentrations. The shake-flask culture of strain PT5(Mmp-T7N) was performed and induced by adding various IPTGs when the cell density reached 0.1 at OD_{600}. After 24 h, medium broth was taken for determination of enzyme activity. Bars: 1, without IPTG; 2, 0.05 mM IPTG; 3, 0.1 mM IPTG; 4, 0.3 mM IPTG; 5, 0.5 mM IPTG; 6, 0.7 mM IPTG; 7, 1.0 mM IPTG; 8, 1.5 mM IPTG. The experiment was done in duplicate. (**B**) Response of the nattokinase production to various induction times. As described in (**A**), the strain was grown and induced by adding 0.5 mM IPTG at various times. The experiment was done in duplicate. Bars: 1, induction at 0.1 of OD_{600}; 2, induction at 0.5 of OD_{600}; 3, induction at 1 of OD_{600}; 4, induction at 2 of OD_{600}; 5, induction at 3 of OD_{600}.

induction times (**Figure 4B**). The production of nattokinase reached a maximum when the culture was induced by IPTG at OD_{600} between 0.1 and 0.5. Beyond that, protein production began to decrease sharply.

Secreted Production of *T. fusca* Endoglucanase E1. To illustrate the general use of the T7 system, plasmid pMT1-E1 was constructed to place the *T. fusca* endoglucanase E1 gene under the regulation of the T7 promoter and the *aprN* signal peptide (**Figure 1E**). Strain PT5(MT1-E1) was constructed to carry a
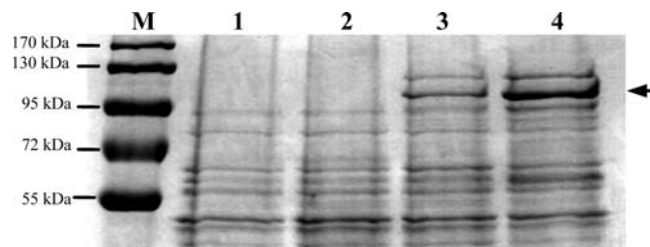

Figure 5. SDS-PAGE analysis of secreted *T. fusca* endoglucanase E1. The shake-flask cultures were performed and induced by adding 0.5 mM IPTG when the cell density reached 0.1 at OD_{600}. After 24 h, medium broth was taken for SDS-PAGE analysis. Lanes: 1, PT5 without IPTG; 2, PT5 with 0.5 mM IPTG; 3, PT5(MT1-E1) without IPTG; 4, PT5(MT1-E1) with 0.5 mM IPTG. The position of *T. fusca* endoglucanase E1 is indicated by an arrow.

genomic copy of the T7 promoter-driven endoglucanase E1 located at *mpr* gene. This was carried out by first transformation of plasmid pMT1-E1 into strain PT5 and by later removal of the selection marker following the method as outlined in **Figure 2A**. Strain PT5(MT1-E1) was then cultured in shake flasks for endoglucanase E1 production. After 24 h, the culture broth was subjected to SDS-PAGE analysis. As shown in **Figure 5**, the production of secreted recombinant endoglucanase E1 could be detected in uninduced or induced culture. With IPTG, the strain was able to secrete endoglucanase E1 with the yield reaching 80 mg/L and the activity toward CMC achieving 105 U/mg.

DISCUSSION

Plasmids are an indispensable tool for achieving high production of recombinant proteins in host cells. However, plasmid instability and plasmid burden are two problems frequently encountered (*18*). In addition, the use of antibiotics as a selective pressure is unfavorable for some applications, whereas it may also lead to the spread of antibiotic-resistant markers to the environment by horizontal transfer (*22*). To address these issues, the T7 expression system was adopted in *B. subtilis*. For chromosomal integration of heterologous genes, a markerless and replicon-free method was then developed (**Figure 2A**). The approach relies on the construction of two flanking FRT sites in the suicidal plasmid, which carries the guide DNA fused with the passenger DNA to be inserted (**Figure 1**). After integration, the antibiotic-resistant gene and plasmid-replication region surrounded by two FRT sites are eliminated by the act of the FLP recombinase provided from a helper plasmid. Accordingly, with this approach, strain PT5 was created to harbor a genomic copy of the spac promoter-regulated T7 gene 1. Moreover, the genes encoding nattokinase and endoglucanase E1 were fused with the T7 promoter and directed into strain PT5 genome to give two "clean" strains (without carrying any selection makers), PT5(Mmp-T7N) and PT5(MT1-E1), respectively.

As illustrated, strains PT5(Mmp-T7N) and PT5(MT1-E1) were able to secrete nattokinase and endoglucanase E1, the production of which increased with the addition of IPTG (**Figures 3** and **5**). This result clearly indicates the functionality of the IPTG-inducible T7 system in *B. subtilis*. Nevertheless, a basal level of target proteins could be found in the absence of IPTG, probably due to less stringency of the spac promoter. In comparison with its counterpart bearing plasmid (PT5/pUKT-NAT), strain PT5(Mmp-T7N) produced a higher level of nattokinase. Indeed, plasmid pUKT-NAT originating from pUB110 has a copy number of around 50−60 copies per cell. Nattokinase produced by strain PT5/pUKT-NAT was found in part unprocessed and retained within the cell cytoplasm (**Figure 3C**). Overall,

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this suggests that multiple copies of plasmid-encoded genes are unnecessary and even adverse for this developed T7 system. This is in sharp contrast to previous work that reported the construction of the T7 system under the control of the *xylA* promoter (*30, 31*). With the use of plasmid-borne genes, proteins of choice were largely secreted upon inhibition of the host RNA polymerase activity by rifampicin, indicating that our developed T7 system exhibits higher efficiency.

Unlike homologous proteins, the secretion of heterologous proteins by *B. subtilis* is frequently inefficient. This has been attributed to plasmid instability, proteolysis, and bottlenecks present in the secretory pathway of proteins (*24, 32*). Our proposed approach excludes the use of plasmids. In particular, it recruits the genes (*wprA* and *mpr*) encoding extracellular proteases for the genomic loci of insertion, thereby resulting in the producer strains deprived of six proteases, replicons, and selection markers. Moreover, the constructed strains exhibited high stability for secretion of the two recombinant proteins after 10 consecutive cultivations. With the illustration of producing two heterologous proteins, the system developed here may have a general use and holds promise for stable and high secretion of recombinant proteins in *B. subtilis*.

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