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Development of high-performance inducible and secretory expression vector and host system for enhanced recombinant protein production

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The production of lipopolysaccharide (LPS)-free recombinant proteins from culture supernatants is of great interest to biomedical research and industry. Due to the LPS-free cell wall structure and the well-defined secretion factor B (SecB)-dependent secretion pathway, Gram-positive bacteria are a superior alternative to *Escherichia coli* expression systems. However, the lack of inducible expression systems for high yields has been a bottleneck. To address this, we developed the pKS81 plasmid, featuring the *uhpT* (glucose-6-phosphate [G6P] transporter) promoter for high expression of recombinant proteins induced by extracellular G6P via a three-component hexose phosphate transport regulatory system (HptARS), the N-terminal SecB-dependent signal peptide sequence for recombinant protein secretion, and the C-terminal 8 × histidine tag for purification by nickel affinity chromatography. We also generated an expression host strain, *Staphylococcus aureus* LAC9, lacks the *uhpT* gene and harmful superantigen and leukotoxin genes, allowing for constitutive HptARS activation by extracellular G6P and increased safety, respectively. Using the pKS81 plasmid, we successfully achieved high yields of prokaryotic (staphylococcal leukotoxin E) and eukaryotic (human annexin A2 protein tagged with mouse IgG1) recombinant proteins, up to 900 mg/L. Our newly established inducible and secretory expression system provides for efficient production and easy purification of LPS-free recombinant proteins, making it valuable for biomedical research and industrial applications.

Keywords Hexose phosphate transport system, Inducible and secretory protein expression system, Extracellular production

A large-scale production of recombinant proteins is important for biopharmaceutical companies and other industries. *Escherichia coli* (*E. coli*) has been the most widely used recombinant protein expression agent due to the ease of genetic manipulation and the availability of various inducible expression vectors that reproducibly produce large protein yields. The main challenges with protein expression and purification in *E. coli* systems are a lack of an efficient secretory system that requires complicated purification processes and results in contamination by lipopolysaccharides (LPS) which is highly toxic to humans[1,2]. In *E. coli*, proteins are synthesized in the cytoplasm and excreted by the secretion factor B (SecB)-dependent type II secretion system by which pre-proteins are carried to the inner membrane and transferred to the periplasmic space where they are folded and excreted by non-specific periplasmic leakage[3,4]. However, many recombinant proteins are trapped in the periplasm[5], which requires enzymatic and/or mechanical disruption for protein purification. During these processes, recombinant proteins are mechanically or enzymatically damaged resulting in functional loss or poor yield[6]. Furthermore, recombinant proteins purified from *E. coli* are heavily contaminated with LPS which can cause secretion of proinflammatory cytokines, inhibition of cell growth, and hyperactivation of immune cells,

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resulting in endotoxic shock or even death[7,8]. Therefore, LPS must be removed from FDA-approved biologics which requires extensive and costly downstream procedures[9,10].

Gram-positive bacteria possess a single layer of cytoplasmic membrane free of LPS and the presence of defined SecB-dependent secretory system which allows the translocation of recombinant proteins into the culture supernatants without LPS contamination, making it an ideal system to produce recombinant proteins for biomedical uses[11,12]. Several Gram-positive bacteria including *Bacillus*, *Lactococcus*, and *Streptomyces* have been used in industry to produce a variety of recombinant proteins[13–16]. However, large-scale production of recombinant protein has been challenging due to the lack of efficient inducible expression systems.

Recently, we characterized the hexose phosphate transport (Hpt) system in *Staphylococcus aureus*[17]. The Hpt system is composed of the three-component regulatory system (HptARS) and the UhpT hexose phosphate transporter. HptA senses extracellular glucose-6-phosphate (G6P) which induces phosphorylation of the HptS histidine kinase, followed by phosphorylation of the HptR transcriptional factor in the HptRS regulatory system. The phosphorylated HptR binds to the *uhpT* gene promoter region (GTTCAGTATTTTGGATAATTTAATAA TTTT) and induces *uhpT* transcription at several hundred-fold to facilitate uptake G6P from the media[17,18].

In this study, we developed an inducible secretory expression vector system by generating a pKS81 plasmid, containing the *uhpT* gene promoter and the SecB-dependent signal peptide sequence, in a *S. aureus* UhpT deletion mutant expression host strain. Using this system, we demonstrated successful expression of prokaryotic and eukaryotic proteins at yields of up to 900 mg/L. These results indicate that the inducible secretory expression vector system established in this study provides for efficient production and easy purification of LPS-free recombinant protein which can be broadly used for biomedical research and industry.

Materials and methods

Bacterial strains, plasmids, DNA constructs, and peptide sequences

The *E. coli*-*S. aureus* shuttle vector, pOS1, was obtained from Dr. Taeok Bae (Indiana University). The DNA sequences of the *uhpT* promoter, the signal peptide sequence of Hlb (UniProtKB: P09978), LukE (UniProtKB: O54081), AnxA2 (UniProtKB: P07355), and mouse IgGFc (UniProtKB: P01868) were chemically synthesized (GenScript Biotech). A bioluminescent reporter plasmid, pLuxABCDE (Caliper) was used to assess the transcriptional activity of the *uhpT* promoter.

Construction of inducible secretory expression vector system, pKS81 and *S. aureus* expression host strain

The synthetic oligonucleotides containing the *uhpT* gene promoter, a signal peptide sequence of Hlb, multiple cloning sites, and the C-terminal 8 × histidine residues were synthesized (GenScript Biotech) and cloned into the EcoRI/NheI restriction sites in the pOS1 plasmid, resulting in a pKS81 plasmid (Fig. 1A).

A modified pMAD-CM-GFP$_{uv}$, a temperature-sensitive shuttle vector system, was used to generate markerless deletion of the *uhpT*, superantigen and leukotoxin genes from *S. aureus* LAC resulting in *S. aureus* LAC9 (Δ*uhpT*, Δ*hlgABC*, Δ*lukFS*, Δ*hla*, Δ*lukGH*, Δ*psm*, Δ*lukDE*, Δ*selq*, Δ*selk*, Δ*selx*) as described previously[17]. Briefly, the upstream and downstream of the target gene fragments were amplified by PCR and cloned into pMAD-CM-GFPuv. The resulting plasmid was electroporated into *S. aureus* LAC using an ECM 630 exponential decay wave electroporation system (BTX®, Harvard Apparatus). The transformed strain was cultured in CY (1% *c*asamino acids, 1% *y*east extract, 100 mM NaCl) agar plate with chloramphenicol (10 μg/mL) at 43 °C using Isotemp™ incubator (Fisher Scientific), which is a non-permissive temperature for the plasmid replication, to promote the first homologous recombination. A single colony was transferred to CY agar plate with chloramphenicol and cultured at 37 °C to promote the second recombination, resulting in a deletion of the target gene. The deletion of the *uhpT*, superantigen, and leukotoxin genes was confirmed by sequencing using a nanopore sequencing kit (Nanopore technologies, UK).

Measuring transcriptional activity of the *uhpT* promoter by bioluminescent signal

To measure the induction of target gene expression by G6P under the control of the *uhpT* promoter, a synthetic DNA fragment containing the *uhpT* gene promoter region was cloned into a promoterless bioluminescent plasmid (pLuxABCDE)[19]. The resulting plasmid was transformed into *E. coli* DH5α, transferred into *S. aureus* RN4220, and subsequently electroporated into *S. aureus* LAC wild type or LAC9. *S. aureus* wild type or LAC9 strains, harboring the constructed plasmid, were cultured in CY broth without or with 2% G6P at 37 °C and bioluminescence was measured using Cytation 5 cell imaging multimode reader (BioTek Instrument). To determine the optimal G6P concentration for inducing maximal target gene expression, LAC9 harboring the constructed plasmid were cultured in CY broth supplemented with varying G6P concentrations (0, 0.5, 1, 2 or 3% w/v), corresponding to approximately 19.2 mM, 38.4 mM, 76.8 mM, and 115.3 mM, respectively, at 37 °C and bioluminescence was measured using the same method.

Expression and purification of staphylococcal leukotoxin E (LukE) and human annexin A2 fused with mouse IgG1 heavy chain (AnxA2-mIgG1) using pKS81 and *S. aureus* expression host strain

Synthetic DNA encoding LukE or AnxA2-mIgG1 was cloned between the BamHI and XhoI sites in the pKS81. The resulting plasmids were transformed into *S. aureus* LAC9 by electroporation. To determine the optimal G6P concentration for maximal LukE expression, the transformed strains harboring pKS81 LukE were cultured in CY broth containing various concentrations of G6P (0, 0.5, 1, 2, or 3% w/v) at 37 °C for 18 h with shaking at 200 rpm using MaxQ™ 4450 Benchtop Orbital Shaker (ThermoFisher scientific). To verify the protein expression in culture supernatants, the samples were centrifuged at 13,500 × g using a microcentrifuge (Eppendorf), supernatant proteins were then concentrated by precipitation with trichloroacetic acid (10%, w/v). The proteins

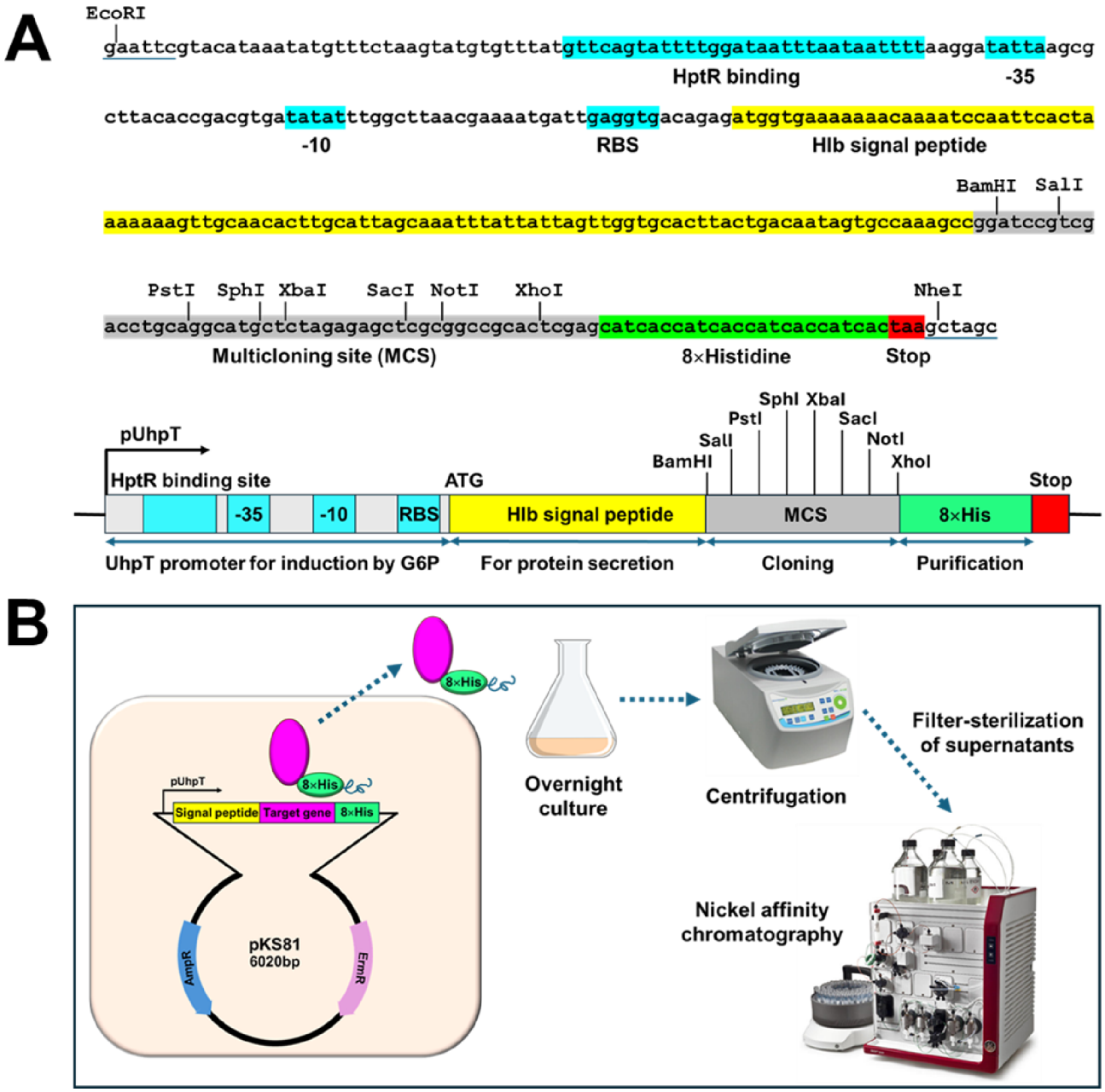

Fig. 1. Construction of an inducible secretory expression system, pKS81. (**A**) The sequence of the HptR binding site, *uhpT* promoter (-35 and -10), ribosome binding site, and N-terminal signal peptide sequence of the Hlb, multicloning sites, and 8 × histidine tag cloned into the pOS1 plasmid. (**B**) A schematic illustration of recombinant protein expression, secretion, and purification process.

were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 125 V for 1.5 h using a XCell SureLock™ Mini-Cell electrophoresis system (Invitrogen) and visualized using Coomassie Blue staining. The image of the stained gel was digitized by the ChemiDoc™ MP imaging system (Bio-Rad Laboratories, Inc) using Image Lab™ Touch Software, version 2.4.0.03, with optimal auto-exposure setting.

For protein purification, LAC9 strains harboring the constructed plasmids were cultured in 50 mL of CY broth containing 2% G6P at 37 °C for 18 h with shaking at 200 rpm. Generally, OD_{600} of overnight culture ranged around 1.8. The culture supernatants were collected by centrifugation at 18,000 × g for 10 min using Sorvall RC6 + superspeed centrifuge, filter-sterilized using 0.45 µm polyethersulfone syringe filter (Corning), and the His-tagged proteins were purified on the Hisprep™ Fast flow 16/10 column (Cytiva) by ÄKTA pure™ chromatography systems (Cytiva) with Unicorn 6.4 software, as suggested by the manufacturer. Purification was conducted at a flow rate of 5 mL/min, 22 °C, with detection at 280 nm and the fraction volume of 5 mL. The elution buffer was exchanged with PBS using Amicon® Ultra Centrifugal filter tube (10 kDa MWCO, Millipore Sigma), and the final volume was adjusted to 3 mL. Protein concentrations were quantified by measuring the

absorbance at 280 nm using a Nanodrop Spectrophotometer (Thermo Fisher Scientific). Purified proteins were analyzed by 12.5% SDS-PAGE and visualized using Coomassie Blue staining, with digitized gel images captured as described above.

Cytotoxicity assay with human leukocytes

A cytotoxicity assay with human leukocytes was performed to verify the biological activity of recombinant LukE expressed in our pKS81 vector system. Blood was obtained by venipuncture from healthy volunteers and informed consent was obtained from the volunteers in accordance with the protocol (18–283), which was reviewed and approved by the Institutional Review Board at Mississippi State University. Human leukocytes were purified by lysing red blood cells with ammonium-chloride-potassium lysing buffer (Invitrogen). Purified human leukocytes were adjusted to 1×10^6/mL in serum free RPMI media. Cells were co-incubated with purified LukE (1 μg), LukD (1 μg, Abcam), or both LukD and LukE for 30 min and then propidium iodine solution (1μM) was added. The fluorescent intensity as an indication of membrane damage was measured using Cytation 5 cell imaging multimode reader (BioTek Instrument).

Statistical analysis

The statistical significance of data for cytotoxicity assay was analyzed by Student t-test using GraphPad Prism version 9.4.1 (GraphPad).

Results

Construction of an inducible and secretory expression vector system and a compatible *S. Aureus* host strain

Synthetic DNA containing the HptR binding sites, the promoter region of the *uhpT* gene (-35 and -10 sites), a ribosome binding site, and the N-terminal secretory signal peptide sequence of the β-hemolysin (Hlb), the multicloning sites, and C-terminal 8 × histidine residues was chemically synthesized and cloned into the pOS1 *E.coli-S. aureus* shuttle vector, resulting in an inducible and secretory expression vector system, pKS81 (Fig. 1A). The resulting pKS81 vector allows for induced expression of the target gene by extracellular G6P and secretion of the expressed protein into the media due to the SecB-dependent Hlb signal peptide sequence. The C-terminal 8 × histidine tag allows for simplified purification of the expressed protein from the supernatant using nickel affinity column chromatography (Fig. 1B).

Since G6P is a highly metabolizable sugar quickly depleted by bacterial metabolism, the transcriptional activation of the *uhpT* promoter by G6P could be temporal, lasting only as long as G6P remains in the culture media. However, since UhpT is the only G6P transporter in *S. aureus*[17], we speculated that a deletion of the *uhpT* gene would conserve G6P in the culture media which could constitutively activate the HptARS system and sustain transcriptional activation from the *uhpT* promoter. In addition, *S. aureus* LAC strain produces superantigens and leukotoxins and the contamination of these toxins could be harmful. To ensure high yield production of recombinant protein and increased safety, we removed the *uhpT*, superantigen, and leukotoxin genes from *S. aureus* LAC wild type strain using a markerless deletion system[17], resulting in *S. aureus* LAC9 strain (Δ*uhpT*, Δ*hlgABC*, Δ*lukFS*, Δ*hla*, Δ*lukGH*, Δ*psm*, Δ*lukDE*, Δ*selq*, Δ*selk*, Δ*selx*). To demonstrate constitutive transcriptional activation of the *uhpT* promoter by extracellular G6P, the *uhpT* promoter was cloned into a promoterless bioluminescent pLuxABCDE plasmid, which was transformed into *S. aureus* LAC wild type and LAC9 strain (Fig. 2A). When *S. aureus* wild type strain was cultured in the CY broth supplemented with G6P, the bioluminescent signal rapidly increased, peaked at 4 h, and then decreased thereafter (Fig. 2B). By contrast, the bioluminescent signal from *S. aureus* LAC9 was sustained throughout a whole culture period. These results indicated that a deletion of the *uhpT* gene resulted in the sustained presence of G6P in the culture media, which constitutively activated the HptARS system to induce continuous transcriptional activation of the *uhpT* promoter. It is noted that no bioluminescent signal was observed from both *S. aureus* wildtype and *ΔuhpT* strains cultured in the CY broth without G6P supplementation. These results indicated that transcriptional activation of the *uhpT* promoter is strictly dependent on the presence of G6P in the culture media.

In addition, to determine the optimal conditions for maximal the *uhpT* promoter activity, LAC9 was cultured with various G6P concentrations (0–3% w/v). The bioluminescence intensity exhibited a dose-dependent response, with 2% and 3% G6P yielding robust and sustained levels of bioluminescent signals (Fig. 2C). This result suggests that 2% and 3% G6P supplementation would be suitable for maximal expression of target protein.

Expression of prokaryotic proteins using an inducible and secretory protein expression vector system

To further confirm the optimal G6P concentration for protein expression, the staphylococcal leukotoxin E (*lukE*) gene was amplified and cloned into the pKS81 plasmid which was transformed into *S. aureus* LAC9. When cultured in CY media with varying G6P concentrations (0–3% w/v), the strain showed a dose-dependent increase in LukE protein expression levels (Fig. 2D), consistent with the dose-dependent *uhpT* promoter activity observed in Fig. 2C. The bioluminescence signals and protein expression levels were similar at 2% and 3% G6P, indicating that 2% G6P was fully sufficient for maximal transcriptional activity and protein expression. Therefore, 2% G6P was selected as the optimal concentration for target protein expression and purifications in the subsequent experiments.

To demonstrate that the inducible and secretory expression system established in this study can produce a large quantity of prokaryotic protein LukE, the supernatant from the strain cultured in the CY media supplemented with 2% G6P (w/v) showed high level of LukE expression at the expected molecular weight of 32.7 kDa which was absent in the culture supernatant from the CY media without G6P (Fig. 3A). A highly pure LukE protein was obtained by nickel affinity chromatography, a yield of up to 15 mg/50 mL of culture.

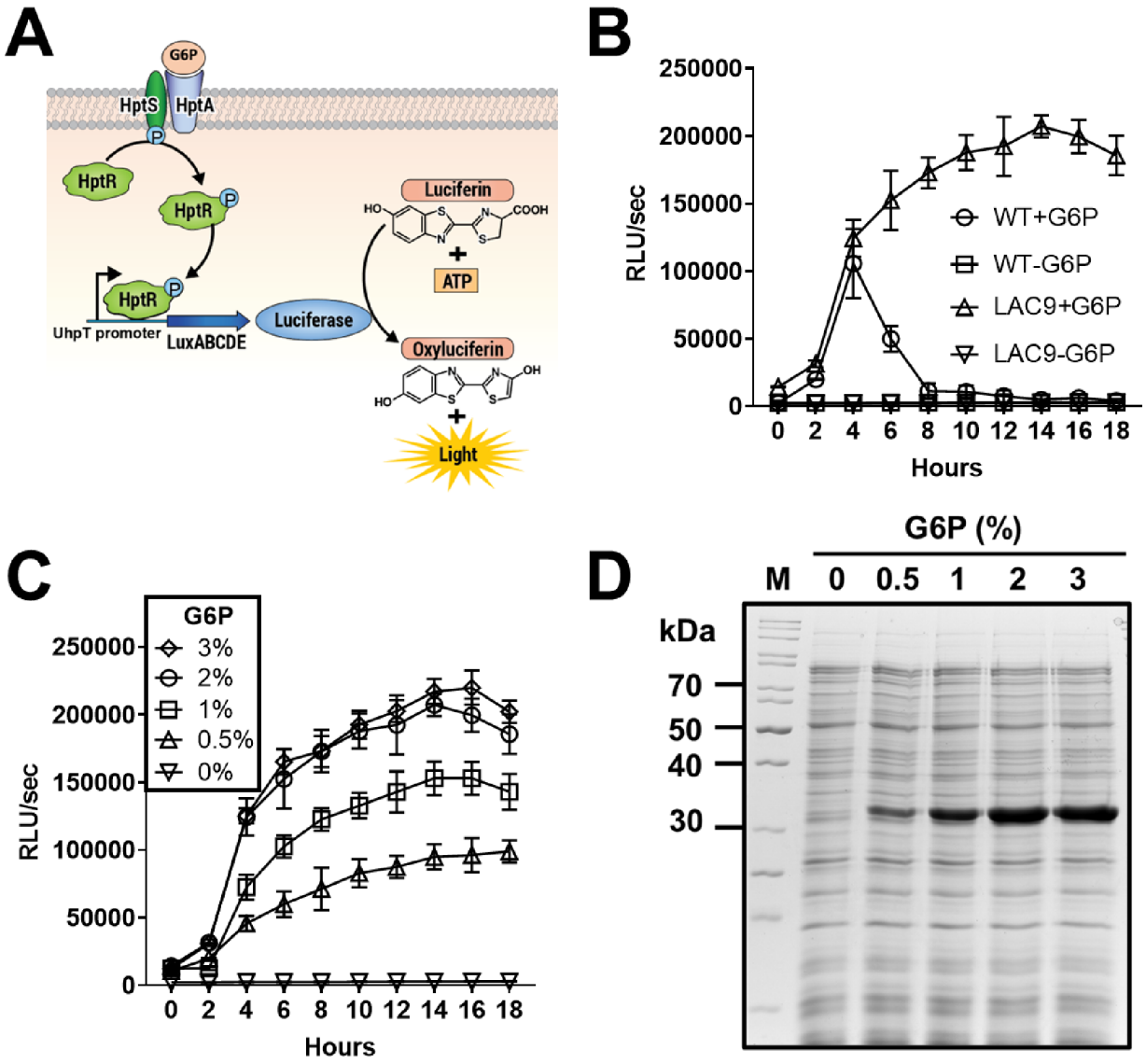

Fig. 2. Analyzing transcriptional activation of the *uhpT* promoter and protein expression by G6P. (**A**) A schematic illustration of bioluminescent signal induced by extracellular G6P via HptARS, three-component regulatory system and the *uhpT* promoter cloned into the promoterless pLuxABCDE plasmid. (**B**) *S. aureus* LAC wild type and LAC9 strains harboring a bioluminescent reporter plasmid pLuxABCDE were cultured in CY broth supplemented without or with 2% G6P. Activation of the *uhpT* gene promoter, indicated by induction of bioluminescent signal, was measured using Cytation 5 cell imaging multimode reader (BioTek). Data shown are the mean ± SEM combined from three independent experiments. (**C**) *S. aureus* LAC9 harboring a bioluminescent reporter plasmid pLuxABCDE were cultured in CY broth supplemented with various concentrations of G6P (0, 0.5, 1, 2, or 3% w/v). Activation of the *uhpT* gene promoter, indicated by induction of bioluminescent signal, was measured using Cytation 5 cell imaging multimode reader (BioTek). Data shown are the mean ± SEM combined from three independent experiments. (**D**) A synthetic DNA fragment encoding LukE was cloned into BamHI and XhoI sites in the pKS81 plasmid which was transformed into *S. aureus* LAC9 strain. Transformed strain was cultured in CY broth supplemented with various concentrations of G6P (0, 0.5, 1, 2, or 3% w/v) for 18 h at 37 °C with shaking at 200 rpm. The proteins in culture supernatant were collected and protein expression was analyzed by SDS-PAGE followed by Coomassie Blue staining. M: Protein marker, lane 1: culture supernatant from CY broth without G6P, lane 2: with 0.5% G6P, lane 3: with 1% G6P, lane 4: with 2% G6P, lane 5: with 3% G6P.

To verify the bioactivity of the purified LukE, we performed a cytotoxicity assay using human leukocytes. The LukE is an S component of the bi-component leukotoxin, which is not cytotoxic by itself but requires an F-component of the bi-component leukotoxin D (LukD) for cytotoxicity[20]. As shown in Fig. 3B, incubation of human leukocytes with LukE alone did not induce any cytotoxicity. By contrast, incubation of human leukocytes with both LukE and LukD (obtained from Abcam) induced a strong cytotoxicity, as indicated by the propidium iodine signal. These results indicated that the LukE expressed and purified from our study was highly pure and

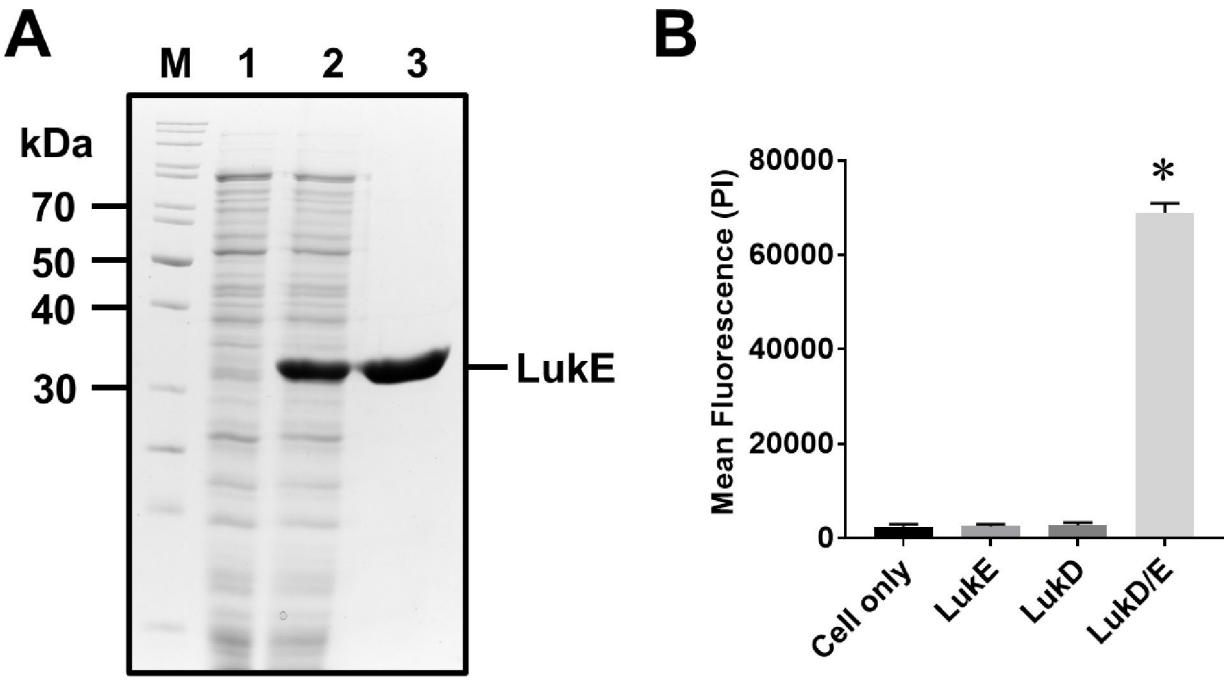

Fig. 3. Biological activity of recombinant LukE expressed and purified from an inducible and secretory protein expression vector system, pKS81. (**A**) A synthetic DNA fragment encoding LukE was cloned into BamHI and XhoI sites in the pKS81 plasmid which was transformed into *S. aureus* LAC9 strain. Transformed strain was cultured in CY broth without or with supplementation of G6P (2%, w/v) for 18 h at 37 ºC with shaking at 200 rpm. The culture supernatant was collected and recombinant LukE protein was purified by the nickel affinity chromatography. Protein expression and purification were analyzed by the SDS-PAGE with Coomassie Blue staining. M: Protein marker, lane 1: culture supernatant from CY broth without G6P, lane 2: culture supernatant from CY broth with G6P, lane 3: purified LukE. (**B**) Human leukocytes (1×10^6 cell/mL) were incubated with purified LukE (1 µg), LukD (1 µg, Abcam), or both LukE/LukD (1 µg each) for 30 min at 37º C. Cytotoxicity as indicated by incorporation of propidium iodine (PI) to the cellular DNA was measured using Cytation 5 cell imaging multimode reader (BioTek). Data shown are the mean ± SEM combined from three independent experiments. Asterisk indicates statistical significance in Student t-test, compared to the results from cell only ($p < 0.001$).

biologically active. It also indicates that the expression host *S. aureus* LAC9 strain did not produce any other harmful bi-component leukotoxins.

Expression of eukaryotic protein using an inducible and secretory protein expression vector system

Initially, we attempted to express AnxA2-mIgG1 using a conventional isopropyl β-d-1-thiogalactopyranoside (IPTG)-inducible pET expression system in *E. coli* BL21(DE3) pLysS. However, we were unable to obtain soluble AnxA2-mIgG1; instead, we produced a low quantity of insoluble protein, rendering it unsuitable for bioassays (Fig. 4A). Additionally, the expression levels were inconsistent across trials, with some experiments yielding no detectable expression in the *E. coli* expression system. We then cloned the gene encoding the human annexin A2 fused with mouse IgG1 Fc (AnxA2-mIgG1) into the pKS81 plasmid which was transformed into *S. aureus* LAC9. The supernatant from the strain cultured in the CY media supplemented with 2% G6P (w/v) showed a high level of AnxA2-mIgG1 expression at the expected molecular weight of 69.1 kDa, while no expression was observed in the culture supernatant from the CY media without G6P (Fig. 4B). A large quantity of pure AnxA2-mIgG1 protein was obtained by nickel affinity chromatography, with a yield of up to 45 mg/50 mL of culture.

Discussion

A lack of LPS and efficient secretion of recombinant proteins are great advantages of protein production in Gram-positive bacteria[12,16,21]. However, the lack of efficient inducible expression systems has been a challenge[22]. In this study, we leveraged the HptARS system in *S. aureus*, a bacterial three-component regulatory system which responds to extracellular G6P and induces transcriptional activation of the *uhpT* promoter by several thousand folds[17]. We also utilized *S. aureus* LAC9 strain lacking the *uhpT* gene as an expression host so that extracellular G6P would not be metabolized by *S. aureus*, and rather, constitutively activate the HptARS system for high-yield production of recombinant protein by the *uhpT* promoter. It is noteworthy that transcriptional activation of the *uhpT* promoter is tightly regulated by G6P as indicated by no bioluminescent signal from *S. aureus* LAC9 cultured in CY media without supplementation of G6P (Fig. 2B). This is because G6P is the only signal molecule that activates the HptARS system. In addition, varying concentrations (0–3%) of G6P significantly influence the

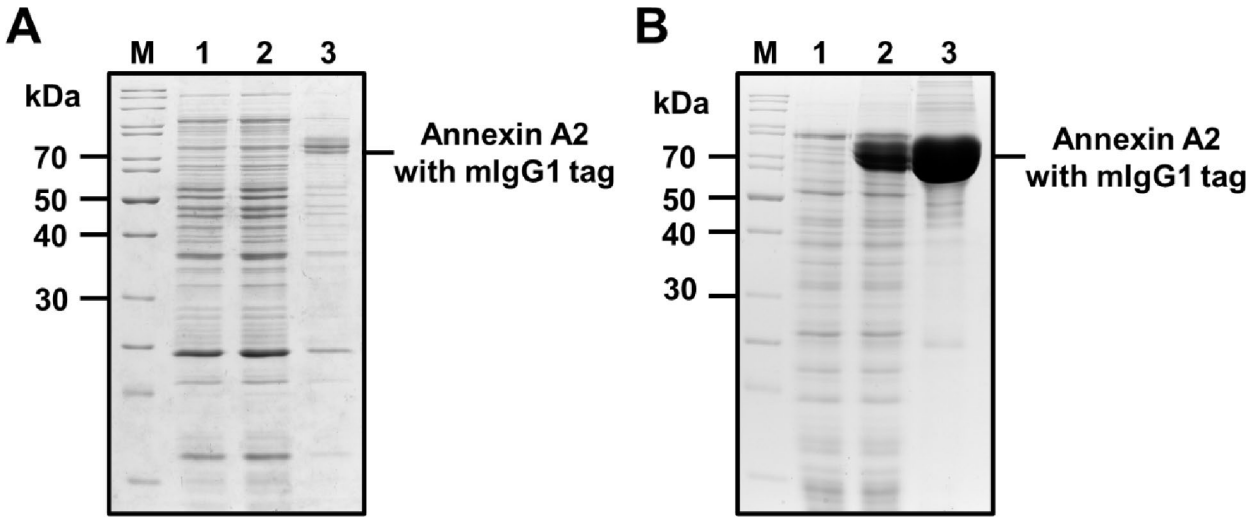

Fig. 4. Enhanced expression and purification of human annexin A2 tagged with mIgG1 by an inducible and secretory protein expression vector system, pKS81. (**A**) Expression of AnxA2-mIgG1in pET expression system using a pET28 vector in *E. coli* BL21 (DE3) pLysS strains with IPTG induction. Protein expression was analyzed by the SDS-PAGE with Coomassie Blue staining. M: Protein marker, lane 1: Uninduced, lane 2: IPTG induced soluble fraction, lane 3: IPTG induced insoluble fraction. (**B**) Expression of AnxA2-mIgG1 using a pKS81vector in *S. aureus* LAC9. Transformed *S. aureus* LAC9 was cultured in the CY broth with or without G6P (2%, w/v) for 18 h at 37° C with shaking at 200 rpm. The culture supernatant was collected and AnxA2-mIgG1 was purified by the nickel affinity chromatography. Protein expression and purification were analyzed by the SDS-PAGE with Coomassie Blue staining. M: Protein marker, lane 1: culture supernatant from CY broth without G6P, lane 2: culture supernatant from CY broth with G6P, lane 3: purified human annexin A2 with mIgG1 tag.

activation of the *uhpT* promoter (Fig. 2C) and the yield of LukE proteins (Fig. 2D) in a dose-dependent manner, with 2% G6P showing optimal induction for high yields. This tight regulation is advantageous for producing toxic proteins or proteins that tend to form inclusion bodies when expressed at high concentrations.

For simple expression and purification of target proteins, we designed an expression vector system to clone the target gene in-frame, fused between the N-terminal signal peptide sequence of *Hlb* gene and a C-terminal 8 × histidine residue sequence. Secretion of proteins in Gram-positive bacteria is mostly mediated by the Sec-dependent pathway in which the N-terminal signal peptide of target protein is recognized by the signal recognition particle (SRP), which transfers the target protein to the cytoplasmic membrane[22–24]. The target protein is then translocated across the cytoplasmic membrane by the Sec translocase and cleaved by the signal peptidase (SPase) at the alanine-X-alanine motif in the signal peptide sequence, resulting in the release of target protein into the culture media[22,24,25]. This provides the added benefit that recombinant proteins retain their native conformation, thereby maintaining their functional properties. Moreover, the concentration of G6P used in the culture has been shown to directly affect the yield of recombinant proteins, underscoring the importance of optimizing this parameter for efficient protein production.

As proof of principle, we expressed both a prokaryotic (LukE) and a eukaryotic protein (AnxA2-mIgG tag) using a pKS81 and demonstrated yields of 15 mg and 45 mg, respectively, from a 50 mL culture supplemented with 2% G6P. Typically, the yield of these recombinant LukE and AnxA2-mIgG in *E. coli* with IPTG inducible system was 5 mg and < 1 mg, respectively. Eukaryotic protein expressions in *E. coli* are often challenging due to factors such as their tendency to form insoluble aggregates withing the cytoplasm, making recovery of functional proteins difficult and inefficient[26–28]. For instance, in our experience, AnxA2-mIgG expressed in *E. coli* primarily partitioned into the insoluble fraction, yielding either a small amount of protein or none at all in different experiments (Fig. 4A). High expression levels can also induce stress responses in the cells, which further reduce protein yields. A study by Sivashanmugam et al.[29] optimized conditions to achieve high yields of recombinant proteins in *E. coli* using high cell density methods, reaching a very high cell density of OD_{600} 10–20 and resulting in 14–34 mg from a 50 mL culture for seven different eukaryotic proteins. In contrast, our expression vector system and the *S. aureus* host strain established in this study have demonstrated higher efficiency in producing both prokaryotic and eukaryotic recombinant proteins, using a cell density of only OD_{600} 1.8. This suggests that our expression system could provide an effective tool for expressing challenging proteins, such as AnxA2-mIgG.

To evaluate the purity and bioactivity of recombinant proteins expressed from the pKS81 system, we tested the cytotoxicity of LukE against human leukocytes. Our results showed that LukE alone did not result in cytotoxicity against human leukocytes but was highly cytotoxic in the presence of LukD, the F component of the bi-component leukotoxin. This indicates that the LukE purified using our system is both highly pure and functionally active.

In conclusion, our newly established inducible and secretory expression system underscores the versability and high efficiency in generating a large amount of highly pure and LPS-free recombinant proteins, both

prokaryotic and eukaryotic, from Gram-positive bacteria, suitable and useful for production of FDA-approved biologics. This system is a robust tool for generating and purifying biologically active proteins, with the added benefit that adjusting G6P concentrations can optimize protein yields, thereby enhancing its applicability in biomedical research and biotechnology.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information file.

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References

1. Rosano, G. L. & Ceccarelli, E. A. Recombinant protein expression in Escherichia coli: advances and challenges. *Front. Microbiol.* **5**, 172. https://doi.org/10.3389/fmicb.2014.00172 (2014).
2. İncir, İ. & Kaplan, Ö. Escherichia coli as a versatile cell factory: advances and challenges in recombinant protein production. *Protein Expr Purif.* **219**, 106463. https://doi.org/10.1016/j.pep.2024.106463 (2024).
3. Mergulhão, F. J., Summers, D. K. & Monteiro, G. A. Recombinant protein secretion in Escherichia coli. *Biotechnol. Adv.* **23**, 177–202. https://doi.org/10.1016/j.biotechadv.2004.11.003 (2005).
4. Johnson, T. L., Abendroth, J., Hol, W. G. & Sandkvist, M. Type II secretion: from structure to function. *FEMS Microbiol. Lett.* **255**, 175–186. https://doi.org/10.1111/j.1574-6968.2006.00102.x (2006).
5. Chen, S., Liu, Z., Chen, J. & Wu, J. Study on improvement of extracellular production of recombinant Thermobifida fusca cutinase by Escherichia coli. *Appl. Biochem. Biotechnol.* **165**, 666–675. https://doi.org/10.1007/s12010-011-9286-z (2011).
6. Slouka, C., Kopp, J., Spadiut, O. & Herwig, C. Perspectives of inclusion bodies for bio-based products: curse or blessing? *Appl. Microbiol. Biotechnol.* **103**, 1143–1153. https://doi.org/10.1007/s00253-018-9569-1 (2019).
7. Heine, H., Rietschel, E. T. & Ulmer, A. J. The biology of endotoxin. *Mol. Biotechnol.* **19**, 279–296. https://doi.org/10.1385/mb:19:3:279 (2001).
8. Bonhomme, D., Cavaillon, J. M. & Werts, C. The dangerous liaisons in innate immunity involving recombinant proteins and endotoxins: examples from the literature and the Leptospira field. *J. Biol. Chem.* **300**, 105506. https://doi.org/10.1016/j.jbc.2023.105506 (2024).
9. Magalhães, P. O. et al. Methods of endotoxin removal from biological preparations: a review. *J. Pharm. Pharm. Sci.* **10**, 388–404 (2007).
10. Mamat, U. et al. Detoxifying Escherichia coli for endotoxin-free production of recombinant proteins. *Microb. Cell. Fact.* **14**, 57. https://doi.org/10.1186/s12934-015-0241-5 (2015).
11. Moreillon, P. & Majcherczyk, P. A. Proinflammatory activity of cell-wall constituents from gram-positive bacteria. *Scand. J. Infect. Dis.* **35**, 632–641. https://doi.org/10.1080/00365540310016259 (2003).
12. Yamane, K., Bunai, K. & Kakeshita, H. Protein traffic for secretion and related machinery of Bacillus subtilis. *Biosci. Biotechnol. Biochem.* **68**, 2007–2023. https://doi.org/10.1271/bbb.68.2007 (2004).
13. Song, A. A., In, L. L. A., Lim, S. H. E. & Rahim, R. A. A review on Lactococcus lactis: from food to factory. *Microb. Cell. Fact.* **16**, 55. https://doi.org/10.1186/s12934-017-0669-x (2017).
14. Souza, C. C., Guimarães, J. M., Pereira, S. D. S. & Mariúba, L. A. M. The multifunctionality of expression systems in Bacillus subtilis: emerging devices for the production of recombinant proteins. *Exp. Biol. Med. (Maywood)* **246**, 2443–2453. https://doi.org/10.1177/15353702211030189 (2021).
15. Anné, J., Maldonado, B., Van Impe, J., Van Mellaert, L. & Bernaerts, K. Recombinant protein production and streptomycetes. *J. Biotechnol.* **158**, 159–167. https://doi.org/10.1016/j.jbiotec.2011.06.028 (2012).
16. Anné, J., Economou, A. & Bernaerts, K. Protein secretion in Gram-positive Bacteria: from multiple pathways to Biotechnology. *Curr. Top. Microbiol. Immunol.* **404**, 267–308. https://doi.org/10.1007/82_2016_49 (2017).
17. Park, J. Y. et al. Characterization of a novel two-component regulatory system, HptRS, the regulator for the hexose phosphate transport system in Staphylococcus aureus. **83**, 1620–1628. https://doi.org/10.1128/iai.03109-14 (2015).
18. Yang, Y. et al. Regulatory mechanism of the three-component system HptRSA in glucose-6-phosphate uptake in Staphylococcus aureus. *Med. Microbiol. Immunol.* **205**, 241–253. https://doi.org/10.1007/s00430-015-0446-6 (2016).
19. Francis, K. P. et al. Monitoring bioluminescent *Staphylococcus aureus* infections in living mice using a novel luxABCDE construct. *Infect. Immun.* **68**, 3594–3600. https://doi.org/10.1128/iai.68.6.3594-3600.2000 (2000).
20. Alonzo, F. 3, Torres, V. J. & rd & The bicomponent pore-forming leucocidins of Staphylococcus aureus. *Microbiol. Mol. Biol. Rev.* **78**, 199–230. https://doi.org/10.1128/mmbr.00055-13 (2014).
21. Guan, C. et al. Construction of a highly active secretory expression system via an engineered dual promoter and a highly efficient signal peptide in Bacillus subtilis. *N Biotechnol.* **33**, 372–379. https://doi.org/10.1016/j.nbt.2016.01.005 (2016).
22. Peng, C. et al. Factors influencing recombinant protein secretion efficiency in Gram-positive Bacteria: signal peptide and Beyond. *Front. Bioeng. Biotechnol.* **7**, 139. https://doi.org/10.3389/fbioe.2019.00139 (2019).
23. Schneewind, O. & Missiakas, D. Sec-secretion and sortase-mediated anchoring of proteins in Gram-positive bacteria. *Biochim. Biophys. Acta* **1843**, 1687–1697. https://doi.org/10.1016/j.bbamcr.2013.11.009 (2014).
24. Freudl, R. Signal peptides for recombinant protein secretion in bacterial expression systems. *Microb. Cell. Fact.* **17**, 52. https://doi.org/10.1186/s12934-018-0901-3 (2018).
25. Zalucki, Y. M. & Jennings, M. P. Signal peptidase I processed secretory signal sequences: selection for and against specific amino acids at the second position of mature protein. *Biochem. Biophys. Res. Commun.* **483**, 972–977. https://doi.org/10.1016/j.bbrc.2017.01.044 (2017).
26. Frenzel, A., Hust, M. & Schirrmann, T. Expression of recombinant antibodies. *Front. Immunol.* **4**, 217. https://doi.org/10.3389/fimmu.2013.00217 (2013).
27. Francis, D. M. & Page, R. Strategies to optimize protein expression in E. Coli. *Curr. Protoc. Protein Sci. Chap* **5** https://doi.org/10.1002/0471140864.ps0524s61 (2010).
28. Huleani, S., Roberts, M. R., Beales, L. & Papaioannou, E. H. Escherichia coli as an antibody expression host for the production of diagnostic proteins: significance and expression. *Crit. Rev. Biotechnol.* **42**, 756–773. https://doi.org/10.1080/07388551.2021.1967871 (2022).
29. Sivashanmugam, A. et al. Practical protocols for production of very high yields of recombinant proteins using Escherichia coli. *Protein Sci.* **18**, 936–948. https://doi.org/10.1002/pro.102 (2009).

Author contributions

S.Y., K.S.S., and J.Y.P conceived the studies, designed experiments, and wrote the manuscript. S.Y., C.K., N.P.,

and P.D. performed experiments and data analyses. J.A.T. wrote and edited the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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