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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Engineering the Tat-secretion pathway of *Bacillus licheniformis* for the secretion of cytoplasmic enzyme arginase

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Abstract

The industrial bacterium *Bacillus licheniformis* has long been used as a microbial factory for the production of enzymes due to its ability to secrete copious amounts of native extracellular proteins and its generally regarded as safe (GRAS) status. However, most attempts to use *B. licheniformis* to produce heterologous and cytoplasmic enzymes primarily via the general secretory (Sec) pathway have had limited success. The twin-arginine transport (Tat) pathway offers a promising alternative for the extracellular export of Sec-incompatible proteins because it transports full, correctly folded proteins. However, compared to the Sec pathway, the yields of the Tat pathway have historically been too low for commercial use. To improve the export efficiency of the Tat pathway, we identified the optimal Tat-dependent signal peptides and increased the abundance of the Tat translocases, the signal peptidase (SPase), and the intracellular chaperones. These strategic modifications significantly improved the Tat-dependent secretion of the cytoplasmic enzyme arginase into the culture medium using *B. licheniformis*. The extracellular enzymatic activity of arginase showed a 5.2-fold increase after these modifications. Moreover, compared to the start strain *B. licheniformis* 0F3, the production of extracellular GFP was improved by 3.8 times using the strategic modified strain *B. licheniformis* 0F13, and the extracellular enzymatic activity of SOX had a 1.3-fold increase using the strain *B. licheniformis* 0F14. This Tat-based production chassis has the potential for enhanced production of Sec-incompatible enzymes, therefore expanding the capability of *B. licheniformis* as an efficient cellular factory for the production of high-value proteins.

Key points

- *Systematic genetic modification of Tat-pathway in B. licheniformis.*
- *Significant enhancement of the secretion capacity of Tat pathway for delivery the cytoplasmic enzyme arginase.*
- *A new platform for efficient extracellular production of Sec-incompatible enzymes.*

Keywords *Bacillus licheniformis* · The Tat pathway · Protein secretion · Microbial cell factory

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Introduction

Gram-positive *Bacillus* species, such as *Bacillus subtilis* and *Bacillus licheniformis*, are important producers of various industrial enzymes including protease, amylase, and lipase for commercial use (Anne et al. 2017). In contrast to gram-negative bacteria that usually secret proteins to the periplasmic space, proteins secreted by gram-positive bacteria are released into the culture medium, facilitating easy and economic downstream purification processes. In addition, these *Bacillus* species possess numerous merits including generally regarded as safe (GRAS) status, rapid growth on inexpensive carbon sources, ease of genetic manipulation, absence of codon bias, and high product yields (Yzturk et al.

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2016). However, most attempts to use *Bacillus* species for the manufacture of heterologous or native intracellular proteins have been met with limited success. These failures are due to a combination of the properties of the secretion pathway, the *Bacillus* cell envelope, quality control proteases, and the cargo proteins themselves (Harwood and Cranenburgh 2008).

In bacteria, two major transport pathways exist for protein translocation across the cytoplasmic membrane, namely the general secretory (Sec) pathway and the twin-arginine translocation (Tat) pathway. In *Bacillus* species, the Sec pathway is the most important transport route responsible for the export of the majority of bacterial proteins, whereas only four proteins (PhoD, QcrA, YkuE, and YwbN) have so far been identified to be exported by the Tat pathway (Goosens et al. 2014). *Bacillus* species possess a minimal Tat system that is simply composed of TatA and TatC subunits. Moreover, there are two parallel TatA-TatC pathways, namely TatAy-TatCy and TatAd-TatCd, and each pathway works independently with its own substrate specificities (Goosens et al. 2013; Jongbloed et al. 2000). TatAd-TatCd is only expressed under low-phosphate conditions with one known substrate, PhoD (Pop et al. 2002), whereas TatAy-TatCy is expressed constitutively and has a broader array of native substrates including YwbN, QcrA, and YkuE (Goosens et al. 2014; Tjalsma et al. 2000).

The Sec pathway transports unfolded proteins, whereas the Tat pathway transports folded or even oligomeric proteins. Furthermore, the Tat pathway enables co-factors such as metals to be incorporated into proteins during folding in the cytoplasm prior to transport (Palmer and Berks 2012). Oligomers can also be transported by the Tat pathway while only one subunit of an oligomeric protein needs the signal peptide to export the whole protein, but the oligomeric structure must be achieved in the cytoplasm and retained during the translocation (Rodrigue et al. 1999). A high proportion of heterologous and cytoplasmic proteins are difficult for the Sec pathway to handle because they fold too rapidly or too tightly (Georgiou and Segatori 2005). Thus, the Tat pathway offers a potentially important alternative to the Sec pathway for the transport of such proteins. There are several examples demonstrating that the Tat pathway can export complex heterologous proteins that are Sec-incompatible, like the tightly folded dihydrofolate reductase with bound methotrexate (Hynds et al. 1998), the green fluorescent protein (GFP) (Santini et al. 2001), and several bio-pharmaceutically relevant human proteins (Alanen et al. 2015). However, the major obstacle for the Tat system to be used industrially is the low flux compared to the Sec system.

Arginase is a manganese-dependent enzyme catalyzing the hydrolysis of L-arginine to produce L-ornithine and urea, and it can be considered responsible for the cyclic nature of the urea cycle (Bewley et al. 1996). This enzyme is widely distributed in organisms as diverse as bacteria, yeasts, plants, invertebrates, and vertebrates (Jenkinson et al. 1996). Many studies claim that arginase could be used to treat arginine-dependent cancers in mammals by decreasing the arginine levels in cancer cells to inhibit their growth (Cheng et al. 2007; Hsueh et al. 2012; Tsui et al. 2009). Moreover, arginase has been used in the industry for L-ornithine production through enzymatic conversion of L-arginine (Zhang et al. 2015). L-ornithine is a non-protein amino acid that plays a significant role in human metabolism, and is used for the treatment of liver diseases, to strengthen the heart, and to boost immunity, which has a sizable market worldwide (Lee et al. 2000).

Similar to most bacterial arginases, the *Bacillus* arginase is a hexamer, and each monomer contains a dimanganese active site (Bewley et al. 1999). In this study, we explored the use of both the Sec and Tat secretion systems of *B. licheniformis* to export the native cytoplasmic enzyme arginase into the culture supernatant. Based on the results, the Sec pathway failed to secrete active arginase, whereas the Tat pathway successfully transported active arginase into the extracellular medium. Therefore, we optimized the Tat pathway through multilayer modulation in *B. licheniformis*, including screening and optimization of signal peptides (SPs) to target arginase to the Tat transporter system and overexpression of SPases, chaperones, and translocase components, to build an effective system for the extracellular production of arginase up to a commercially acceptable level. This is the first systematic modification of the Tat pathway in *Bacillus* species for high-yield extracellular enzyme production, and this efficient platform is also expected to be valuable for the extracellular production of other Sec-incompatible proteins.

Materials and methods

Strains, plasmids, and cultivation conditions

The main strains in this study are listed in Table 1, and all the stains and plasmids used in this study are shown in Table S1. *Escherichia coli* DH5α was used for the construction of recombinant plasmids. *B. licheniformis* was used to express the recombinant proteins. The plasmid pHY-300PLK was used for gene overexpression, and the plasmid T2(2)-Ori was used for gene deletion and integration in *B. licheniformis*.

All organisms were grown on LB liquid medium or LB agar plates containing antibiotics when necessary (20 mg/L kanamycin, 20 mg/L tetracycline). The arginase production medium was LB supplemented with 400 μM $MnSO_4$. Low-phosphate defined medium (LPDM) was used when genes were fused with the signal peptide PhoD, and this medium

Table 1 The main strains used in this study

Strains	Characteristics	Source
0F3	DW2 (Δ*spo0F*; Δ*bprA*; Δ*aprE*; Δ*pgsC*)	Cai et al. (2016)
0F4	Integration overexpression of *sipV*	This study
0F5	Integration overexpression of *sipW*	This study
0F6	Integration overexpression of *sipS*	This study
0F7	Integration overexpression of *sipT*	This study
0F8	Deletion of *hrcA* in 0F3	This study
0F9	Integration overexpression of Tat*AdCd*	This study
0F10	Integration overexpression of Tat*AyCy*	This study
0F11	Deletion of Tat*AdCd* and Tat*AyCy*	This study
0F12	Integration overexpression of *sipS*;deletion of *hrcA*	This study
0F13	Integration overexpression of *sipS* and Tat*AdCd*;deletion of *hrcA*	This study
0F14	Integration overexpression of *sipS* and Tat*AyCy*;deletion of *hrcA*	This study

contains 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4{\cdot}7H_2O$, 27 mM KCl, 7 mM sodium citrate·$2H_2O$, 50 mM Tris–HCl (pH 7.5) supplemented with 0.6 mM KH_2PO_4, 2 mM $CaCl_2{\cdot}2H_2O$, 1 mM $FeSO_4{\cdot}7H_2O$, 400 μM $MnSO_4{\cdot}H_2O$, 4.5 mM glutamic acid, 780 mM tryptophan, 860 mM lysine, and 1% (w/v) glucose. For arginase and sarcosine oxidase (SOX) production, the cells were grown at 37 °C, 250 rpm for 48 h. For GFP production, the cells were grown in LPDM at 37 °C, 250 rpm for 24 h.

Construction of gene overexpression strains

Using the PhoD signal peptide as an example, the gene construction procedures were performed based on the previously reported method (Cai et al. 2016). The P43 promoter from *B. subtilis* 168, the PhoD signal peptide, the arginase gene *rocF* (Gene ID 16053535), and *amyL* terminator from *B. licheniformis* DW2 (Liu et al. 2018) were amplified and fused by splicing overlap extension (SOE)-PCR. Then, the fused fragment was ligated with the pHY300PLK vector through homologous recombination by ClonExpress® One-Step Cloning Kit (Vazyme, China), and the resulting recombinant plasmid was confirmed by PCR and DNA sequencing. The successfully constructed vector was named pHY-PhoDsp-*rocF*. The pHY-PhoDsp-*rocF* vector was then electro-transferred into *B. licheniformis* 0F3 to construct the arginase overexpression strain, named 0F3/pHY-PhoDsp-*rocF*. The other signal peptide overexpression vectors including YwbNsp, AmiAsp, MdoDsp, TorAsp, DmsAsp, QcrAsp, and YkuEsp were also constructed with using the strategy employed for PhoDsp. For GFP production, the *gfp* gene was fused with the signal peptide PhoDsp to generate the recombinant plasmid pHY-PhoDsp-GFP. For SOX production, the *sox* gene was fused with the signal peptide YwbNsp to generate the recombinant plasmid pHY-YwbNsp-SOX.

Gene deletion in *B. licheniformis*

The *hrcA* gene deletion strain was constructed according to the method we reported previously (Cai et al. 2016). The upstream and downstream homologous regions of *hrcA* were amplified and fused by SOE-PCR. The fusion fragment was inserted into T2(2)-Ori by homologous recombination to generate the gene deletion vector T2-Δ*hrcA*. This deletion vector T2-Δ*hrcA* was electro-transferred into *B. licheniformis* 0F3 to obtain positive transformants. The positive transformants were cultured in LB liquid medium containing 20 μg/mL kanamycin for several generations at 45 °C, and the culture solution was spread on plates to obtain single-crossover transformants. The selected transformants were transferred into LB medium at 37 °C without kanamycin for several generations, and kanamycin-sensitive colonies were further verified by colony PCR to attain the double-crossover strain named 0F8.

Gene integration in *B. licheniformis*

The construction procedure for the gene integrated strains was similar to the method described for gene deletion in the previous section. The integration of the *sipS* gene encoding SPase I is used as an example. The P43 promoter, *sipS* gene, and the *amyL* terminator were fused and inserted into T2(2)-Ori, forming the integrated vector T2-*sipS*. The resulting vector was electro-transferred into the host, and the *sipS* gene was integrated into the chromosome using the double-crossover procedure. The construction method for the other integration strains followed the same procedure.

Enzymatic assay of arginase

Enzyme activities were determined by Sakaguchi test. First, 0.2 g α-naphthol and 5 g urea were dissolved into 100 mL 95% ethanol to prepare Sakaguchi reagent A. Sakaguchi

reagent B was prepared with 5 mL 10 M NaOH and 2 mL 2% NaClO. A reaction mixture containing 10% L-arginine (pH 9.0) and 2 mL fermentation supernatant was incubated at 37 °C for 1 h, and then, the reaction was terminated by incubation at 100 °C for 10 min.

The reaction solutions were diluted 5 times, and 2% arginine solution was used as the negative control. A drop of diluent was dripped on to filter paper, and then, the Sakaguchi reagent A and B were dripped on the same spot successively. Finally, after the filter paper was completely dry, the spots were observed for the development of color. A pink to red color indicates the presence of arginine in the sample.

SDS-PAGE of extracellular proteins

The fermentation broth was harvested at 24 h, 36 h, and 48 h, respectively, by centrifugation. The supernatant was concentrated tenfold, and aliquots comprised of 10 μl of the extracellular proteins mixed with the loading dye were subjected to SDS-PAGE. SDS-PAGE was performed using a 4% acrylamide stacking gel and 12% acrylamide resolving gel.

HPLC-ELSD for L-ornithine analysis

To measure arginase enzyme activity, 40% L-arginine (pH 9.0) and 1 mL fermentation broth mixtures were incubated at 37 °C for 10 min and then held at 100 °C for 10 min to terminate the reaction. The L-ornithine content in the reaction mixture was measured using HPLC-ELSD. In this study, one unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of L-ornithine/min at 37 °C.

Post reaction, the samples were diluted 200-fold and filtered through a 0.22-μm PES Syringe Filter (Biosharp, China). L-arginine and L-ornithine were analyzed using an HPLC system (Agilent 1260, USA) equipped with the Column Ultimate® Amino Acid Plus (Welch Technologies, 4.6 × 300 mm, 5 μm). The mobile phase consisted of 0.3% heptafluorobutyric acid.

Analysis of fluorescence intensity

The fermentation broth was harvested by centrifugation, and fluorescence intensity of the supernatant was measured with a Multi-Mode Microplate Reader (SpectraMax iD3; Molecular Devices). An excitation wavelength of 480 nm and an emission wavelength of 520 nm were used to determine the relative fluorescence intensity of GFP in the LPDM medium supernatant.

SOX enzymatic assay

To measure SOX enzymatic activity, a volume of 100 μL supernatant of fermentation broth was added into 3 mL color developing agent containing 1 mM 4-aminoantipyrine, 6 mM phenol, 200 U/L horse radish peroxidase (HRP), and 20 mM Tris–HCl, pH8.0. As a control, 100 μL LB medium was used with the same reagents. To start the reaction, 150 μL 0.2 M sarcosine was added to the mixture. The reaction reagent was incubated in a 37 °C water bath for 5 min, and then moved into a boiling water bath for 3 min to stop the reaction. The OD_{500} of final reaction mixture was determined by spectrometric measurement.

Results

The Tat pathway is capable of transporting active arginase into the medium

Although the Sec pathway has been widely used to transport biotechnologically relevant proteins with high yield, there are many heterologous proteins, as well as some native cytoplasmic proteins, that fail to be exported by Sec-dependent SPs partly because of folding considerations (Lee et al. 2006). On the other hand, the Tat pathway has been shown to export a complex array of proteins extracellularly. To determine which pathway was suitable for translocation of arginase, we selected nucleic acid sequences for one canonical Sec signal peptide (SacCsp) (Wei et al. 2015) and two Tat SPs (YwbNsp (Liu et al. 2014) and PhoDsp (Pop et al. 2002), respectively) from *B. licheniformis*, and individually fused them to sequences encoding the N-terminus of arginase to examine which transport system could effectively export the active enzyme. A strong promoter (P43) and ribosomal binding site UTR12 from a previous study (Xiao et al. 2020) were used to improve expression of these hybrid SPs with the arginase gene *rocF* from *B. licheniformis.* The fusion constructs were cloned in *E. coli,* and expressed in the previously constructed protein production strain *B. licheniformis* 0F3 which has deletions of two extracellular protease genes *aprE* and *bprA*, the γ-PGA synthesis gene *pgsC*, and a gene essential for sporulation, *spo0F* (Cai et al. 2016). The amount of secreted arginase in the culture supernatant was determined using SDS-PAGE (Fig. 1A). It was observed that all three SPs were able to deliver arginase (Mw 32.5 kDa) into the extracellular medium. Two bands were seen for the arginase delivered by SacCsp, and the upper band may be the arginase with uncleaved SP. To evaluate the enzymatic activity, the Sakaguchi test (Fig. 1B) was performed. This test is used to detect the presence of arginine that reacts with the Sakaguchi reagent, thereby forming a red-colored complex (Joshi 2006). It was found that the arginase delivered by Tat SPs YwbNsp and PhoDsp exhibited activity for the conversion of arginine, but the arginase delivered by Sec SP SacCsp was inactive. The intracellular activity of the SacCsp-fused arginase was observed to be significantly low, as depicted in Figure S1. This finding suggests that the SacCsp protein facilitated the

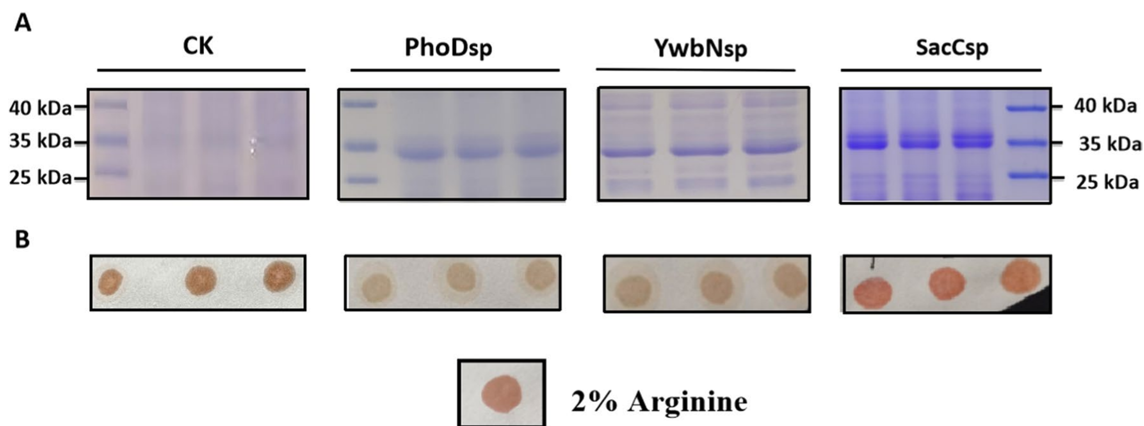


Fig. 1 Comparison of transport efficiency of Sec pathway vs. Tat pathway for arginase in *B. licheniformis*. **A** SDS-PAGE of extracellular arginase. The molecular weight of the arginase RocF is 32.4 kDa. The PhoDsp and YwbNsp represent arginase produced via the Tat pathway, and SacCsp represents arginase produced via the Sec pathway. CK, the stain 0F3/pHY-*rocF* harboring arginase without a SP was used as the negative control. **B** Visualization of enzymatic activity of arginase using Sakaguchi test. A pink to red color indicates the presence of arginine in the sample (no activity). The experiments were performed in triplicate of different cultures

secretion of the majority of arginase into the extracellular environment. These findings suggest that both the Sec and Tat pathways can secrete arginase into the media, but only the enzymes exported by the Tat pathway are active.

Screening of homologous and heterologous Tat signal peptides (SPs) for the secretion of cytoplasmic proteins in *B. licheniformis*

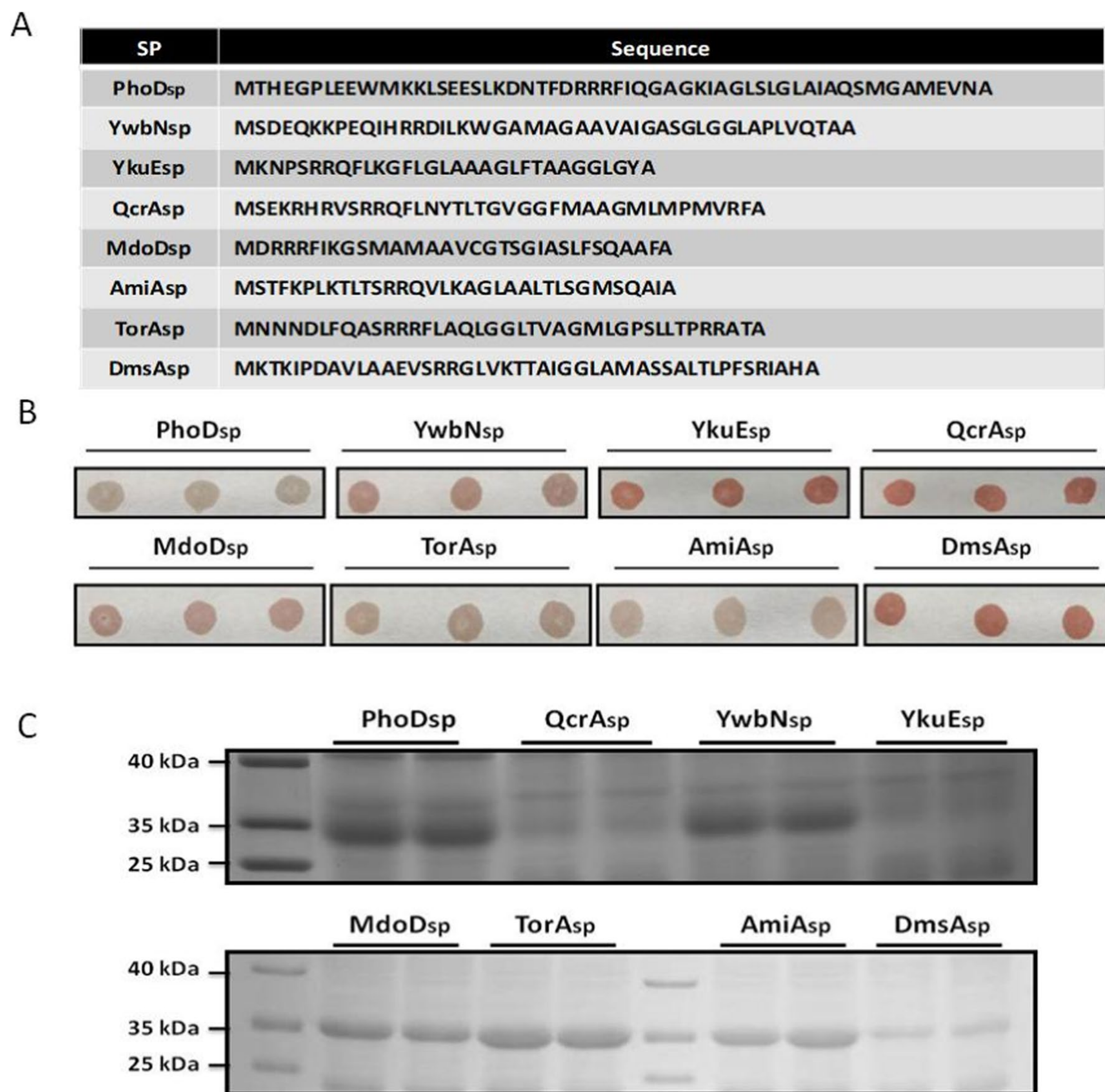
Tat-dependent SPs are similar to Sec-type SPs, and both sets of SPs are comprised of three distinct domains: an N-terminal positively charged domain (N-domain), hydrophobic core domain (H-domain), and more polar C-terminal domain (C-domain) containing the cleavage site for signal peptidases (Mendel et al. 2008). However, the Tat SPs contain a well-conserved twin-arginine (RR) motif for specific targeting to the Tat translocases (Robinson et al. 2011). In addition, the H-domain of Tat SPs is typically less hydrophobic than that of Sec-specific SPs, and Tat SP sequences tend to be longer than their Sec counterparts, with average lengths of 38 and 24 amino acids, respectively (Cristobal et al. 1999). Four Tat SPs from *B. licheniformis* (PhoDsp, YwbNsp, QcrAsp, and YkuEsp) and four Tat SPs from *E. coli* (TorAsp, DmsAsp, AmiAsp, and MdoDsp) were fused to arginase and screened for extracellular arginase activities in *B. licheniformis*. The fusion of enzymes with DmsAsp from *E. coli* and YkuEsp and QcrAsp from *Bacillus* did not demonstrate any extracellular activity (Fig. 2B). The SDS-PAGE analysis of extracellular arginase revealed minimal amounts of arginase present in the extracellular media (Fig. 2B). In contrast, the remaining five SPs successfully transported active arginase into the culture media (Fig. 2).

Secretion efficiency of the Tat pathway can be enhanced via SPase overexpression

Both Sec- and Tat-dependent pathways require SPase I to release the signal peptide from the mature protein upon translocation (Anne et al. 2017). *B. licheniformis* contains four chromosomally encoded SPase I genes, namely *sipS*, *sipV*, *sipT*, and *sipW*. In this study, we integrated an extra copy of these four SPase I genes regulated by the P43 promoter into the *B. licheniformis* chromosome, respectively. The two SPs PhoDsp and AmiAsp using the Tat translocases TatAdCd and TatAyCy, respectively, were utilized to investigate the impact of SPase overexpression on secretion efficiency. HPLC analysis revealed that translocation efficiency in both AmiAsp and PhoDsp could be enhanced by overexpressing any of the four SPases (Fig. 3). However, the response differed slightly between the two SPs. In the case of PhoDsp, overexpression of SipW, SipT, and SipS resulted in ~ 1.8-fold increase in extracellular arginase activity. On the other hand, AmiAsp-mediated translocation of arginase and the overexpression of SipS led to a 3.8-fold increase in arginase activity. Overall, the data indicated that SipS could effectively cleave the Tat SPs and facilitate the export of active protein.

Overexpression of *B. licheniformis* Tat translocases improves Tat export capacity

Bacillus species possess two parallel Tat translocases, namely TatAdCd and TatAyCy, which exhibit distinct cargo specificities. Both Tat translocases have been experimentally validated for their activity in *E. coli*. TatAdCd has been demonstrated to recognize the signal peptide $TorA_{sp}$ (Barnett et al. 2009),



SP	Sequence
PhoDsp	MTHEGPLEEWMKKLSEESLKDNTFDRRRFIQGAGKIAGLSLGLAIAQSMGAMEVNA
YwbNsp	MSDEQKKPEQIHRRDILKWGAMAGAAVAIGASGLGGLAPLVQTAA
YkuEsp	MKNPSRRQFLKGFLGLAAAGLFTAAGGLGYA
QcrAsp	MSEKRHRVSRRQFLNYTLTGVGGFMAAGMLMPMVRFA
MdoDsp	MDRRRFIKGSMAMAAVCGTSGIASLFSQAAFA
AmiAsp	MSTFKPLKTLTSRRQVLKAGLAALTLSGMSQAIA
TorAsp	MNNNDLFQASRRRFLAQLGGLTVAGMLGPSLLTPRRATA
DmsAsp	MKTKIPDAVLAAEVSRRGLVKTTAIGGLAMASSALTLPFSRIAHA

Fig. 2 Screening of extracellular arginase delivered by different Tat SPs. **A** The list of SPs used in this study. **B** Visualization of enzymatic activity of arginase using Sakaguchi test. A pink to red color indicates the presence of arginine in the sample (no activity). Triplicates of different cultures were conducted. **C** SDS-PAGE of extracellular arginase. The molecular weight of the arginase RocF is 32.4 kDa. Duplicates of different cultures were conducted

while TatAyCy is capable of recognizing three SPs: $DmsA_{sp}$, $MdoD_{sp}$, and $AmiA_{sp}$ (Barnett et al. 2009). To investigate the impact of overexpressing these translocases on the exportation of Tat transported proteins, we introduced an additional copy of the *tatAdCd* or *tatAyCy* operon independently into the *B. licheniformis* chromosome. The secretion efficiencies of proteins with $PhoD_{sp}$ and $TorA_{sp}$ were evaluated in the TatAdCd overexpression strain, denoted as 0F9, while the $YwbN_{sp}$ and $AmiA_{sp}$ were assessed for the TatAyCy overexpression strain, named 0F10. Increased abundance of TatAdCd led to 2.6-fold and 2.2-fold enhancement in export capacity for $TorA_{sp}$ and $PhoD_{sp}$, respectively (Fig. 4A). Concurrently, overexpression of TatAyCy resulted in a 3.5-fold and 3.7-fold increase in export capacity for arginase with $AmiA_{sp}$ and $YwbN_{sp}$, respectively (Fig. 4B). Furthermore, we generated strain 0F11 by deleting both *tatAdCd* and *tatAyCy* from the genome of strain 0F3. The deletion of the Tat pathway reduced the secretion of arginase fused with all four Tat SPs, although not completely abolished (Fig. 4). In a previous study, Snyder et al. observed that GFP fused with PhoDsp could be secreted into the medium even in a *tatAdCd* and *tatAyCy* double deletion ancestral strain of *B. subtilis* (Snyder et al. 2014). The authors therefore proposed the existence of an unidentified export pathway and/or export regulator in the ancestral strain of *B. subtilis*. We speculate that *B. licheniformis* 0F3 might possess a similar export pathway to that of the ancestral strain of *B. subtilis*.

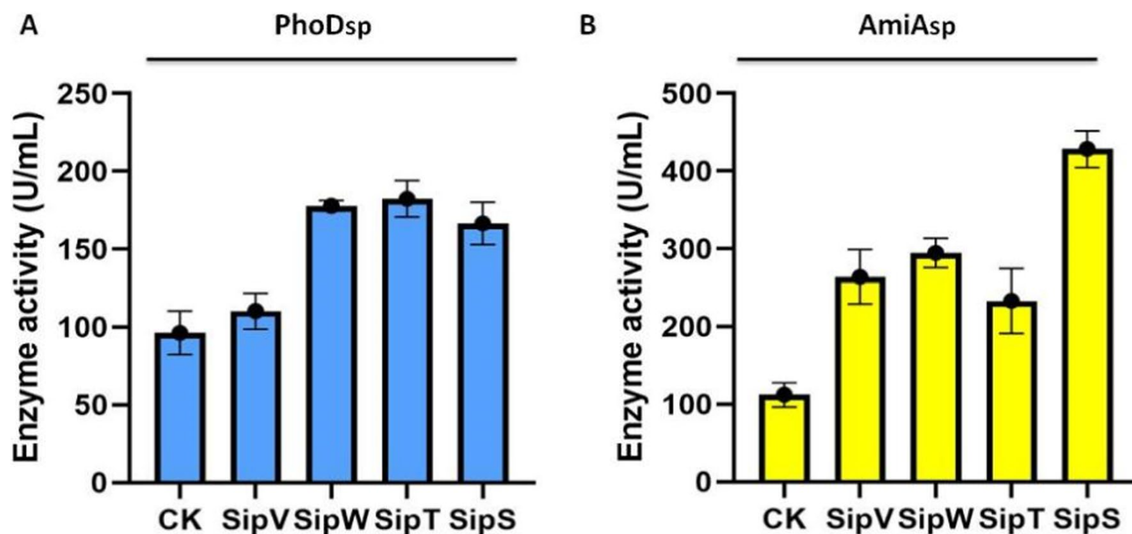


Fig. 3 Signal peptidase overexpression could effectively enhance the secretion efficiency of arginase via the Tat pathway. **A** Extracellular arginase activity conducted by PhoDsp; CK, *B. licheniformis* 0F3 harboring the plasmid pHY-PhoDsp-*rocF*. **B** Extracellular arginase activity conducted by AmiAsp. CK, *B. licheniformis* 0F3 harboring the plasmid pHY-AmiAsp-*rocF*. Results are the averages ± standard deviations from three separate experiments

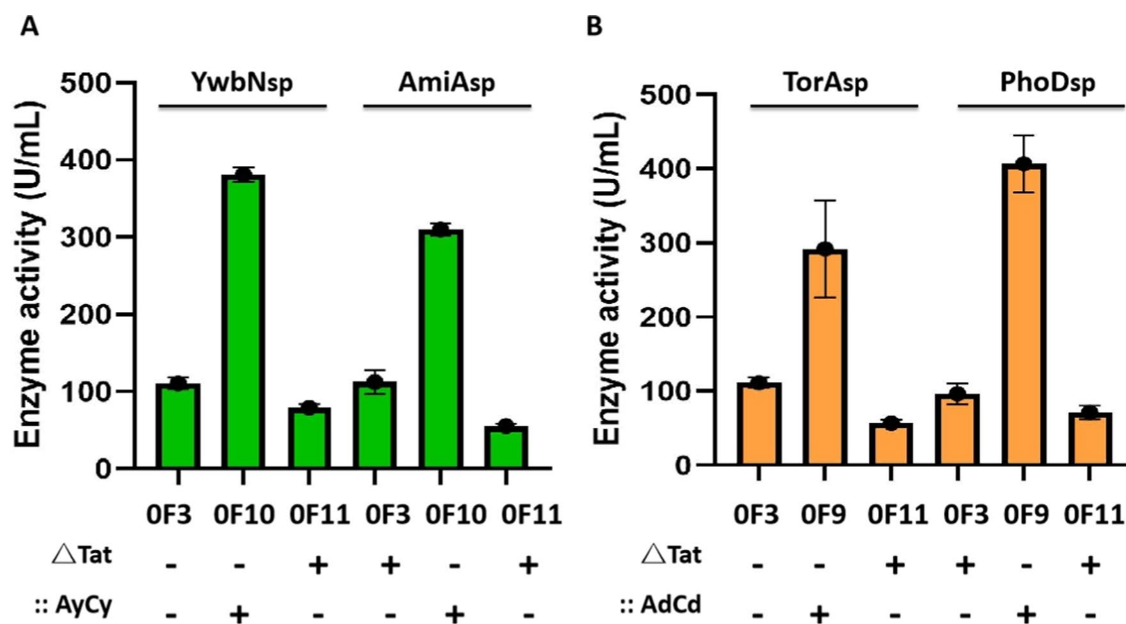


Fig. 4 Increased abundance of Tat translocases enhanced secretion efficiency of active arginase. **A** Extracellular activity of arginase secreted via TatAdCd. **B** Extracellular activity of arginase secreted via TatAyCy. Results are the averages ± standard deviations from three separate experiments

Improvement of extracellular arginase activity by overexpression of intracellular molecular chaperones

The cargo proteins delivered by the Tat pathway need to be properly folded within the cells to be effectively transported across the cell membrane. Interactions with cytoplasmic chaperone networks may be required to prevent aggregation or assist in the assembly of certain Tat substrates. GroEL-GroES and DnaK-DnaJ-GrpE are among the most important chaperone complexes in *Bacillus subtilis*, and these two operons are co-regulated by the heat-inducible transcription repressor HrcA (Mogk et al. 1997). A previous study has shown that the inactivation of HrcA enables overexpression

of both chaperone complexes leading to increased protein solubility (Yuan and Wong 1995). Therefore, we deleted *hrcA* gene in *B. licheniformis* to generate the strain *B. licheniformis* 0F8 and investigated the effect of overexpression of GroEL-GroES and DnaK-DnaJ-GrpE on extracellular arginase production. After the deletion of *hrcA* gene, the transcription levels of the operons *groELS* and *dnaKJ* had a 1.2-fold and a 1.8-fold increase, respectively (Fig. S2), and the extracellular activity of arginase improved by 2–threefold using different SPs (Fig. 5). In comparison to the strain 0F3, the intracellular activities of arginase delivered by PhoDsp and AmiAsp exhibited a decrease in the strain 0F8 (Fig. S1). This finding further supports the notion that the deletion of HrcA can enhance the efficiency of arginase delivery through the Tat pathway.

Combinatorial optimization of Tat secretion capacity of *B. licheniformis*

Based on our results, the effective modifications of *B. licheniformis* 0F3 to boost secretion capacity include overexpression of the signal peptidase SipS, increased expression of Tat translocases, and the deletion of HrcA for enhanced expression of chaperone complexes. To examine the combinatorial effects of these three valid strategies for enhancing extracellular protein production, we first integrated an extra copy of *sipS* into the HrcA-deletion strain *B. licheniformis* 0F8 to generate the strain *B. licheniformis* 0F12, and then integrated the two parallel Tat translocases TatAdCd and TatAyCy onto the chromosome of 0F11 independently, resulting in the strains *B. licheniformis* 0F13 and 0F14. We then examined arginase activity recovered from the medium when expressing the protein with the various Tat SPs in each of the strains. The results showed that the overexpression of SipS concurrent with HrcA deletion had little effect on the secretion capacity for enzymatically active arginase by all four SPs (Fig. 5). However, the integrations of TatAdCd and TatAyCy operons on the genome of 0F12 were both beneficial for the secretion of enzymatically active arginase. Compared to the original strain 0F3, the extracellular enzymatic activities of arginase in the final strains 0F13 and 0F14 had 2.8-fold, 3.7-fold, 4.8-fold and 5.2-fold increases with PhoDsp, TorAsp, AmiAsp, and YwbNsp, as the SPs, respectively (Fig. 5).

Use of the progressively modified strains for the secretion of other Sec-incompatible proteins

While the Sec pathway can export the green fluorescent protein (GFP), it does so in an inactive, improperly folded form (Feilmeier et al. 2000). On the other hand, the properly folded and active GFP has been produced via the Tat system in both *E. coli* (Santini et al. 2001) and *B. subtilis* (Snyder et al. 2014). In order to test the adaptability of the progressively modified *B. licheniformis* for Sec-incompatible proteins, the GFP gene fused with the signal peptide PhoDsp was overexpressed in the parental strain *B. licheniformis* 0F3 and the final strain *B. licheniformis* 0F13, respectively. The extracellular fluorescent intensities were measured in the supernatant of the cells after 24-h, 36-h, and 48-h incubation (Fig. 6A). The extracellular

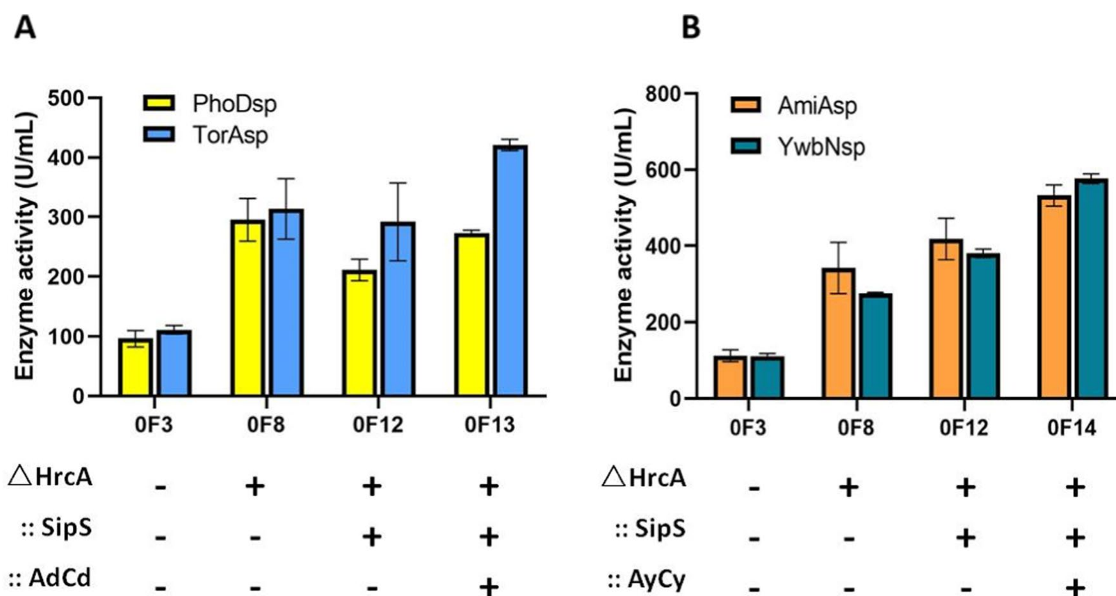


Fig. 5 Progressive modification of *B. licheniformis* for optimized extracellular arginase production. **A** Strains with SPs PhoDsp and TorAsp. **B** Strains with SPs AmiAsp and YwbNsp. Results are the averages ± standard deviations from three separate experiments

fluorescent intensity increased 3.8 times in the strain 0F13 compared with the parental strain 0F3 after 48-h incubation.

Sarcosine oxidase (SOX) catalyzes the oxidative demethylation of sarcosine and forms formaldehyde, glycine, and hydrogen peroxide. Together with the other enzymes, this enzyme has been used in clinics for determination of creatinine, which is an important parameter to estimate the function of kidneys (Suzuki 1994). SOX from *Bacillus* sp. BSD-8 was expressed in *B. licheniformis*. Similar to arginase, SOX could be transported into the medium by both Sec SP $ScaC_{sp}$ and Tat SP $YwbN_{sp}$, but the active enzyme could only be transported by the Tat pathway (Fig. S3). In this study, the SOX gene fused with the signal peptide $YwbN_{sp}$ was overexpressed in the parental strain *B. licheniformis* 0F3 and the final modified strain *B. licheniformis* 0F14, respectively. SDS-PAGE was performed to examine the extracellular production of SOX. Results showed that the secretion capacity of SOX from the progressively modified strain 0F14 improved significantly compared to the parental strain (Fig. 6B), and the extracellular enzymatic activity of SOX from 0F13 also increased by 30% compared to 0F3 (Fig. 6C). These results suggest that the progressively modified strains *B. licheniformis* 0F13 and 0F14 have the potential for enhanced extracellular production of Sec-incompatible proteins.

Discussion

Extracellular production of active enzymes and properly folded proteins are necessary to realize the potential of bacteria as biocatalysts and commercial viability. In this study, we examined and optimized a Tat-based secretion system for the overexpression of a number of proteins that normally cannot be extracellularly produced at high levels in the GRAS strain *Bacillus licheniformis*.

Arginase from *Bacillus* is a cytoplasmic cofactor-containing multimeric enzyme that has strong commercial potential in its use to convert L-arginine to L-ornithine. However, a limitation to commercial production is the ability to produce active enzyme in a recoverable fashion. In this study, we examined the Sec and Tat pathways as possible routes to extracellularly produce arginase to enhance recovery. Our findings indicated that both the Tat and Sec pathways can facilitate the export of arginase into the culture medium, but only the Tat-mediated transport of arginase exhibited enzymatic activity. The Sec pathway is responsible for transporting unfolded proteins that necessitate assembly in the extracellular environment. Given that *Bacillus* arginase is a hexamer relying on Mn^{2+} at its active site, the inactivity observed in arginase exported through the Sec pathway could arise from the inability to incorporate the essential cofactor Mn^{2+} or to assemble into the oligomeric structure. Similar cases have been reported where GFP can be translocated via both Sec and Tat pathway but is only fluorescent in the periplasm when exported by Tat pathway (Feilmeier et al. 2000; Santini et al. 2001). High-level extracellular expression of arginase in *B. licheniformis* was achieved by screening optimal Tat-dependent SPs and modifying the Tat secretion pathway. Five SPs (YwbNsp, PhoDsp, TorAsp, AmiAsp, and MdoDsp) from *E. coli* and *B. licheniformis* were selected to secrete active arginase into the supernatant. Many reports have shown that *B. subtilis* Tat translocases are active in *E. coli* and able to recognize *E. coli* Tat SPs

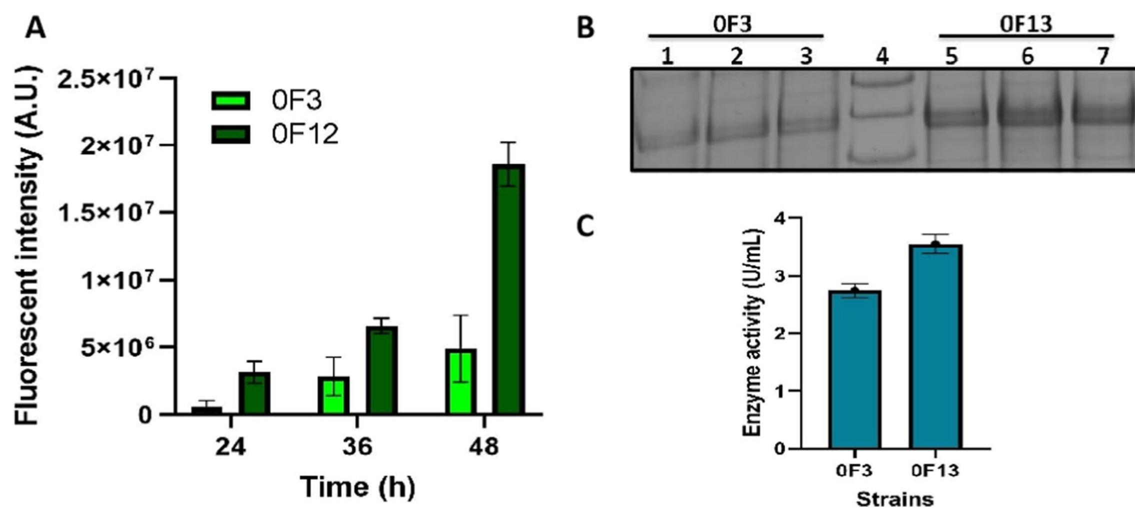


Fig. 6 Capacity of the progressively modified strains 0F12 and 0F13 for secretion of GFP and SOX. **A** Extracellular fluorescence intensity of *B. licheniformis* strains harboring GFP genes. **B** SDS-PAGE of extracellular SOX. The Mw of SOX is 43.1 kDa. Lane 1–3: 0F3, lane 4, ladder: 65 kDa, 45 kDa, 35 kDa. **C** Extracellular enzymatic activity of SOX. Results are the averages ± standard deviations from three separate experiments

(Barnett et al. 2009; Meissner et al. 2007; Monteferrante et al. 2012), and this study demonstrates that Tat SPs from *E. coli* are also functional in *B. subtilis*.

To further improve the secretion efficiency of the Tat pathway in *B. licheniformis*, we overexpressed SPases, increasing the abundance of two parallel Tat translocases TatAdCd and TatAyCy, respectively, and enhanced the expression levels of intracellular molecular chaperones. *B. licheniformis* contains four closely related type I SPases (SipS, SipT, SipW, and SipV); our results suggested that overexpression of SipS could significantly enhance the secretion efficiency via both Tat translocases TatAdCd and TatAyCy. Previous research has shown that SipV plays a major role in the Sec pathway, and overexpression of SipV can enhance the secretion of nattokinase through the Sec pathway (Cai et al. 2016). However, SipV is less efficient to release the enzymes fused with Tat SPs, suggesting that these four SPases have different substrate specificity. Previous research has demonstrated that expression of additional copies of the *tatABC* genes from either a plasmid or the chromosome in *E. coli* could significantly enhance the levels of Tat-targeted protein export (Browning et al. 2017; Matos et al. 2012). Similar to *E. coli*, the low level of expression of Tat translocases in the membrane for native *B. licheniformis* is also the limiting factor for the export yield of extracellular proteins via the Tat system. Yao et al. reported that the inactivation of HrcA in *B. subtilis* resulted in a 1.42-fold increase of the extracellular enzymatic activity of α-amylase secreted via the Sec pathway (Yao et al. 2019). Wu et al. reported similar results when expressing an anti-digoxigennin single-chain antibody in *B. subtilis* (Wu et al. 1998). This study confirmed the hypothesis that the deletion of HrcA could also enhance the secretion ability for the Tat pathway.

Finally, we combined the effective enhancements to optimize the secretion capacity of the Tat pathway for arginase and generated the progressively modified strains *B. licheniformis* 0F12 and 0F13. The results showed that the extracellular enzyme activity (577 U/mL) of the final strain 0F13 harboring arginase fused with YwbNsp had a 5.2-fold increase compared to the original production strain *B. licheniformis* 0F3 (Fig. 5B). Moreover, the progressively modified strains could also effectively boost the secretion ability of the Tat pathway for other Sec-incompatible proteins, such as GFP and SOX. In this study, we built a high-efficiency platform using the Tat pathway to produce the cytoplasmic enzyme arginase extracellularly, demonstrating an attractive alternative for the use of *B. licheniformis* as a cell factory for the production of extracellular proteins that are incompatible with the Sec-transport system.

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Author contribution Y.J., J.L., Y.L, L.L., and Y.W. constructed all the recombinant strains; P.L. and P.X. did the enzymatic analysis; Y.J. and J.L. analyzed the data; Y.J., Q.W., and J.L. generated figures, tables, and artworks; Q.W. and Y.J. wrote the original draft of the manuscript; C.T.N. and S.C. edited the manuscript; Q.W. and C.Z. supervised the project; all authors discussed the results and made substantial contributions to the final version.

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Data availability All data generated during this study are included in this published article and its supplementary information files.

Declarations

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

Conflict of interest The authors declare no competing interests.

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