SUPPLEMENTARY INFORMATION

A modular secretion platform in *Bacillus subtilis* enables scalable production of protein-glutamine glutaminase

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Supplementary note S1. Detailed design of platform modules

Promoter modules

As a general design criterion, promoter-derived modules amplified from *Bacillus subtilis* genomic DNA were reconstructed according to their native chromosomal context rather than as minimal promoter fragments. Genomic promoter parts were defined from the upstream regulatory region up to the native start codon (ATG) of the corresponding locus, thereby preserving the promoter-proximal 5′ context associated with each element. Sequence boundaries were established using the *B. subtilis* 168 reference genome (NC_000964.3) in combination with the NCBI Sequence Viewer. All part sequences are detailed in Table S2.

Pgrac100 was included as a strong inducible promoter in our system. This module was inherited from the pHT1469 destination vector, where it is combined with the *Escherichia coli* lac operator and therefore can be de-repressed by IPTG addition. Pgrac100 is derived from the strong heat-responsive *groESL* promoter of *B. subtilis* and contains optimized regulatory features, including modifications in the UP element and in the -35 and -15 regions, originally introduced to enhance recombinant protein accumulation. Thus, beyond Pgrac relevance as a benchmark from previous *Bacillus Cp*PGG studies, Pgrac100 provided an experimentally validated inducible module (Tjalsma et al. 2000; Vitikainen et al. 2001; Phan et al. 2015).

PSecA promoter controls expression of *secA* locus from *B. subtilis*, which encodes the ATPase subunit of the Sec translocase and is active across the logarithmic and stationary phases (Herbort et al. 1999).

PYvyD promoter corresponds to the promoter region of the *yvyD* locus from *B. subtilis*, which is associated with stationary-phase ribosome hibernation and promotes the formation of 100S ribosome dimers (Drzewiecki et al. 1998). Because the native sequence contained an internal *Bsa*I site, PYvyD* version was generated by modification of one nucleotide (from T to C) to make it compatible with Golden Gate assembly. Specific change was made taking into account that the sequence continued to code for the same amino acid.

Pveg was included to broaden the promoter library beyond the limited set previously tested for *Cp*PGG expression. Unlike Pgrac100, PSecA, or PYvyD, Pveg was selected not because of a prior direct report in *Bacillus Cp*PGG production, but because it is one of the best-characterized strong constitutive promoters in *B. subtilis*. It controls expression of the *veg* gene, a vegetative-growth-associated gene linked to biofilm and sporulation-related physiology and is widely used in *Bacillus* engineering due to its robust constitutive activity (Lam et al. 1998; Radeck et al. 2013; Guiziou et al. 2016).

Signal peptide modules

To maintain a uniform signal peptide-coding sequence interface across the modular library, all signal peptide modules were designed with a common 3′ terminal extension (5′-GCGGCG-3′) encoding two alanine residues. This short Ala-Ala addition served as a standardized C-terminal boundary for all signal peptide modules. In addition to preserving module interchangeability, the terminal nucleotides contributed to the Golden Gate overhang used at the SP-GOI junction. Alanine residues were selected for this extension as a conservative solution for the cleavage-site-proximal region, avoiding the introduction of arbitrary residues at the signal peptide boundary while preserving a sequence context broadly compatible with signal peptide processing (Tjalsma et al. 2000; Fu et al. 2007; Zhang et al. 2020)

AmyQ was selected as the main Sec-pathway reference signal peptide because it represents the classical *Bacillus* secretion strategy. In our platform, the sequence used was **amyQ***, the improved

signal peptide version present in pHT1469. The original AmyQ signal peptide derives from the secreted α-amylase of *Bacillus amyloliquefaciens* (Palva et al. 1981).

YdeJ belongs to the Tat pathway, which is able to export folded proteins or multimeric complexes, in contrast to the Sec pathway, which mainly translocates unfolded polypeptide chains. YdeJ corresponds to the signal peptide of a putative lipoprotein from *B. subtilis* that contains a conserved twin arginine motif (RR motif), which directs the protein to Tat-pathway (Tjalsma et al. 2000).

GlmU was selected as the second Tat-type signal peptide. Biologically, GlmU is a twin-arginine signal peptide derived from the *B. licheniformis glmU* locus, associated with a putative bifunctional UDP-N-acetylglucosamine pyrophosphorylase/glucosamine-1-phosphate N-acetyltransferase. In the present study, GlmU* refers to the improved GlmU-R variant reported by Niu et al. (2019), in which the third residue was changed from asparagine to arginine, a modification associated with improved secretion performance.

Coding sequence modules

The coding-sequence library comprised the reporter gene mCherry and the protein-glutaminase homologs *Cp*PGG and *Cc*PGG. For both PGG modules, the native signal peptide sequence was removed so that secretion depended exclusively on the upstream signal peptide module provided by the platform. The native stop codon was also omitted to maintain compatibility with the downstream assembly context, since translation termination was already encoded in the vector backbone downstream of the His-tag sequence. Both PGG coding sequences were codon-optimized for expression in *B. subtilis* before synthesis and cloning. In contrast, the mCherry reporter sequence was used without codon optimization and retained its native stop codon.

Backbone and maintenance vector adaptations

The plasmid **pGGAselect** (part of the NEBridge® Golden Gate Assembly system; New England Biolabs, Ipswich, MA, USA) was used as a **maintenance plasmid** for cloning the PCR amplified modules prior to final assembly. Before use, a restriction-ligation step was performed to remove two undesired *Bsa*I sites from the multiple cloning site, generating **pGGAselect***. Because the multiple cloning site contains two external *Eco*RI sites, *Eco*RI digestion released a 142 bp fragment carrying the unwanted *Bsa*I sites. The linearized plasmid was purified, religated, and transformed into *E. coli* DH5α. Correct reconstruction of pGGAselect* was confirmed by DNA sequencing.

The shuttle vector *Bacillus subtilis*/*Escherichia coli* high-level expression vector **pHT1469** was selected as the **destination backbone** for final construct assembly. The vector contains the strong IPTG-inducible P*grac*100 promoter, the amyQ*signal peptide, a 8xHis tag followed by a terminator, a chloramphenicol resistance gene (*B. subtilis* selection), an ampicillin resistance gene *(E. coli* selection), ColE1 origin of replication, *repA* replication gene, and *lacI* repressor gene. The vector is structurally and segregationally stable, using the theta-mode of replication within *B. subtilis.* Backbone was modified by site-directed mutagenesis (SDM) to remove a non-specific *Bsa*I site within the ampicillin resistance gene. Overlapping primers #001-#002 (Table S1) were designed to mediate the change of a single nucleotide (from a thymine to a guanine) to eliminate the restriction site. Specific change was made considering that the sequence continued to code for the same amino acid. The obtained PCR product was loaded to an agarose gel before and after 1 h incubation at 37 ºC with *Dpn*I to digest the template DNA. After digestion, bands were observed at the expected size. PCR product was directly transformed to *E. coli* DH5α and purified using QIAprep Spin Miniprep Kit. Afterwards, DNA sequencing results verified mutation incorporation. To obtain the final **pHT1469*** backbone, plasmid was modified to remove the promoter and the signal peptide included in pHT1469, and to include specific terminal *Bsa*I sites. Thus, PCR amplification was conducted using primers #006 and #007. Afterwards, the PCR product was digested using *Bam*HI (NEB), ligated, transformed and kept in *E. coli* DH5α maintenance strain.

Table S1. Primers used in this study.

Primer name	Sequence	Notes
#001	CGTGGGTC**G**CGCGGTATCATTGCAG	Primer pair required for site-directed mutagenesis experiment to eliminate the *Bsa*I site from *Amp*^{R} gene in pHT1469. In bold an enlarged letter, the modified nucleotide for unspecific *Bsa*I site lost.
#002	ATACCGCG**C**GACCCACGCTCACCG	
#006	CATATGGATCCGGTCTCCCATCACCATCACCATCACC	Primer pair required for **Pb1.1** obtention. In underlined letters *Bam*HI restriction-site, in grey shading *Bsa*I restriction-site, and in colour shading the respective overhangs.
#007	CATATGGATCCGGTCTCCTTGGTACCAAGCTAATTCCGGT	
#009	CCGCATGAATTCGGTCTCCCCAAAGGAGGTAAGGATCACTAGAAA	Primer pair required for **Pgrac100** obtention. In underlined letters *Eco*RI restriction-site, in grey shading *Bsa*I restriction-site, and in colour shading the respective overhangs.
#010	CCGCATGAATTCGGTCTCCCATTGATCCTTCCTCCTTTATATGG	
#011	CATATTGAATTCGGTCTCCCCAAGAGGAGAGCTTGGACATCG	Primer pair required for **PSecA** obtention. In underlined letters *Eco*RI restriction-site, in grey shading *Bsa*I restriction-site, and in colour shading the respective overhangs.
#055	CCGCATGAATTCGGTCTCCCATTTATAACGCTCCTCTATCATCACAC	
#013	CCGCATGAATTCGGTCTCCCCAAGATCAATTGG**C**CTCTTTCTCTTTTC	Primer pair required for **PYvyD*** obtention. In underlined letters *Eco*RI restriction-site, in grey shading *Bsa*I restriction-site, in colour shading the respective overhangs, and the modified nucleotide for *Bsa*I site lost is enlarged. In yellow shading the unmodified unspecific *Bsa*I site.
#056	CCGCATGAATTCGGTCTCCCATTCAAAGAACGCCTCCCTTTTATTAAG	
#062	CATATTGAATTCGGTCTCCCCAAGGAGTTCTGAGAATTGGTATGCC	Primer pair required for **Pveg** obtention. In

#063	CCGCATGAATTCGGTCTCCCATTGCATCCACCTCACTACATTTATTG	underlined letters *Eco*RI restriction-site, in grey shading *Bsa*I restriction-site, and in colour shading the respective overhangs.
#015	CCGCATGAATTCGGTCTCCAATGATTCAAAAACGAAAGCGGACAG	Primer pair required for **amyQ*** obtention. In underlined letters *Eco*RI restriction-site, in grey shading *Bsa*I restriction-site, in red letters the sequence that encodes two alanine, and in colour shading the respective overhangs.
#018	CCGCATGAATTCGGTCTCCCGCCGCAGCTGATGCTTTTGTAATCGGCA	
#016	CCGCATGAATTCGGTCTCCAATGAAGAAACGCAGAAAGATATGTT	Primer pair required for **YdeJ** obtention. In underlined letters *Eco*RI restriction-site, in grey shading *Bsa*I restriction-site, in red letters the sequence that encodes two alanine, and in colour shading the respective overhangs.
#017	CCGCATGAATTCGGTCTCCCGCCGCCGTACATCCAGCAAGCAAAATC	
#033	CATATCGAATTCGGTCTCCGGCGGATTCAAATGGCAAC	Primer pair required for ***Cp*PGG** obtention. In underlined letters *Eco*RI restriction-site, in grey shading *Bsa*I restriction-site, and in colour shading the respective overhangs.
#035	CCGCATGAATTCGGTCTCTGATGAAAGCCGCAGCTGCTAA	
#022	CATATCGAATTCGGTCTCCGGCGatggtgagcaagggcgaggag	Primer pair required for **mCherry** obtention. In underlined letters *Eco*RI restriction-site, in grey shading *Bsa*I restriction-site, and in colour shading the respective overhangs.
#021*	CCGCATGAATTCGGTCTCCGATGttacttgtacagctcgtccatgc	
#003	CATATGTAAGATTTAAATGCAACCG	Primers used for expression device sequencing (pHT1469)
#004	CTTTTCAGTTGCAGACAAAG	
#027	TACCGAACTTGGCCGTAACC	Primers used for module sequencing in maintenance plasmid (pGGAselect*)
#028	CCCTTAAACGCCTGGTGCTA	

Table S2. Plasmid, promoter, signal peptides, and gene module sequences. In lower-case letters *Eco*RI restriction-sites, in blue *Bam*HI restriction-site, in grey shading the *Bsa*I terminal sites, in red the extra nucleotides, the respective overhangs are underlined, and the specific nucleotide modifications are enlarged.

Pgrac100	gaattcGGTCTCCCCAAAGGAGGTAAGGATCACTAGAAAATTTTTTAAAAAATCTCTT GACATTGGAAGGGAGATATGTTATTATAAGAATTGCGGAATTGTGAGCGGATAACAA TTCCCATATAAAGGAGGAAGGATCAATGGGAGACCgaattc
PSecA	gaattcGGTCTCCCCAAGAGGAGAGCTTGGACATCGTCCGTCAGAAACGCTTTAATTT AAAGCCGATGGATAGTGAAGAAGCGATCTTGCAAATGAATATGCTCGGCCATAATTT CTTTGTTTTCACAAATGCGGAAACAAACCTTACAAATGTCGTGTACCGCAGAAATGA CGGGAAATATGGCTTAATTGAACCGACTGAATAATGAAGAGAAGCCTTCCGTGATGT CCGCGGAAGGTTTTTGTTTTTCTTATTTGCAAATTCTTTGGAAATAACAAAAGGTAT GATATGATAATGAGAGGTATACATGGACTAGTAAATTATTTATACATGCCTCTAAAATA GGCGTGTGATGATAGAGGAGCGTTATAAATGGGAGACCgaattc
PYvyD*	gaattcGGTCTCCCCAAGATCAATTGG**C**CTCTTTCTCTTTTCCCTCTCATGAGTTCTGTG AGTATTTAAAGGAACATTTTCTGATTCATTATAGAAAATGGATGCTGTCTATTCATCAA TGTATGGAACCCTTTTTAATCAATTAGGCGTGTGTGAGGTATTTGTTTCGTTCAATCA GCATATACATATACCTCCGAACCGCCAATAACAGAGCAAATACAAACAAAATTCGACA AAGTTCACTGAATTTTCACAAAAGATTTATGTTTCAGCAGGAATTGTAAAGGGTAAA AGAGAAATAGATACATATCCTTAATAAAAGGGAGGCGTTCTTTGAATGGGAGACCga attc
Pveg	gaattcGGTCTCCCCAAGGAGTTCTGAGAATTGGTATGCCTTATAAGTCCAATTAACAG TTGAAAACCTGCATAGGAGAGCTATGCGGGTTTTTTATTTTACATAATGATACATAATT TACCGAAACTTGCGGAACATAATTGAGGAATCATAGAATTTTGTCAAAATAATTTTAT TGACAACGTCTTATTAACGTTGATATAATTTAAATTTTATTTGACAAAAATGGGCTCGT GTTGTACAATAAATGTAGTGAGGTGGATGCAATGGGAGACCgaattc
amyQ*	gaattcGGTCTCCAATGATTCAAAAACGAAAGCGGACAGTTTCGTTCAGACTTGTGCT TATGTGCACGCTGTTATTTGTCAGTTTGCCGATTACAAAAGCATCAGCTGCGGCGGG AGACCggatcc
YdeJ	gaattcGGTCTCCAATGAAGAAACGCAGAAAGATATGTTATTGCAATACTGCCCTGCT GCTTATGATTTTGCTTGCTGGATGTACGGCGGCGGGAGACCgaattc
GlmU*	gaattcGGTCTCCAATGGAT**AGG**AGGGATAATGGAGGCCAATACATGGATAAGCGGT TTGCAGTTGTGTTAGCAGCTGGTCAAGGAACAAGAATGAAATCAAAGCTATATAAA GTTCTTCATCCTGCGGCGGGAGACCgaattc
mCherry	gaattcGGTCTCCGGCGATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCA AGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTT CGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAA GCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTC AGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACT TGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGA CGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCTTGCAGGACGGCGAGTTCATC TACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAA GAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCC CTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGAC GCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCT ACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTG GAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGT ACAAGTAACATCGGAGACCgaattc
*Cp*PGG	GGTCTCCGGCGGATTCAAATGGCAACCAAGAAATCAACGGCAAAGAAAAACTGAG CGTCAATGACAGCAAACTGAAAGATTTTGGCAAAACAGTTCCGGTCGGCATTGATG AAGAAAACGGCATGATTAAAGTCAGCTTTATGCTGACAGCGCAGTTCTATGAAATCA

	AACCGACAAAAGAAAACGAGCAGTATATTGGCATGCTGAGACAAGCAGTCAAAAAT GAATCACCGGTCCACATTTTTCTGAAACCGAATTCAAACGAAATCGGCAAAGTTGA AAGCGCATCTCCGGAAGATGTTCGCTACTTTAAAACAATCCTGACGAAAGAAGTCA AAGGCCAGACAAATAAACTGGCATCAGTTATTCCGGATGTTGCGACACTGAATAGCC TGTTTAATCAGATCAAAAATCAAAGCTGCGGCACATCAACAGCATCATCACCGTGCA TTACATTTAGATATCCGGTTGATGGCTGCTATGCAAGAGCACATAAAATGAGACAGAT CCTGATGAACAATGGCTACGATTGCGAAAAACAATTTGTCTACGGCAATCTGAAAGC ATCAACAGGCACATGCTGCGTTGCATGGTCATATCATGTTGCAATTCTGGTCAGCTAT AAAAACGCATCAGGCGTTACAGAAAAGCGCATTATTGATCCGTCACTGTTTTCAAGC GGACCGGTTACAGATACAGCTTGGAGAAATGCATGCGTTAATACATCATGCGGCTCA GCATCAGTTTCAAGCTATGCAAATACAGCAGGCAATGTTTATTATCGCAGCCCGTCA AATAGCTATCTGTATGATAATAACCTGATCAACACAAACTGCGTGCTGACAAAATTTT CACTGCTGTCAGGCTGCTCACCGTCACCGGCACCGGATGTTAGCAGCTGCGGCTTT CATCAGAGACC
*Cc*PGG	gaattcGGTCTCCGGCGGTTAATTCATGCTCAGATTCAGCAGCAAATCAAGATCCGAA TCTGGTTGCAAAAGAAAGCAACGAAATCGCCATGAAAGATTTTGGCAAAACAGTTC CGGTTGGCATCGAAAAAGAAGATGGCAAATTCAAAATCAGCTTCATGGTTACAGCA CAGCCGTATGAAATTGCAGATAGCAAAGAAAACGCAGGCTATATTAGCATGATTAGA CAAGCGGTCGAAAATGAAACACCGGTTCATGTTTTTCTGAAAGCGAACACAAACAA AATCGCGAAAGTTGAAAAAGCGACAGCAGATGATATCCGCTACTTTAAAACGGTGT TCAACAAAGAAGAACGCGGAGATTCAAAGAAAGCGGTTTCAGTTATTCCGAATCTT GCAACACTGAACAGCCTGTTTACACAGATTAAAAACCAAGCATGCGGCACATCAAC AGCATCATCACCGTGCATTACATTTAGATATCCGGTTGATGGCTGCTATGCAAGAGCA CATAAAATGAGACAAATTCTGCTGAACGCAGGATACGATTGCGAAAAACAATTTGTC TATGGCAATCTGAGAGCATCAACAGGCACATGCTGCGTTTCATGGGTTTATCATGTT GCAATTCTGGTCAGCTTTAAAAACGCATCAGGCATTGTCGAAAAGCGCATTATTGAT CCGTCACTGTTTTCATCAGGACCGGTTACAGATAGCGCTTGGAGAGCAGCATGCAC AAATACATCATGCGGCTCAGCATCAGTTTCAAGCTATGCAAATACAGCAGGCAATGT TTATTATCGCAGCCCGTCAGGCAGCCTGCTTTATGATAATAACTATGTCAATACGAACT GCGTCCTGAACATTTTTAGCTCACTGTCAGGCTGCTCACCGTCACCGGCACCGTCAG TTGCAAGCTGCGGCTTTCATCAGAGACCgaattc
pHT1469*	GGTCTCCCATCACCATCACCATCACCATCACTAACGTCCCCGGGGCAGCCCGCCTAAT GAGCGGGCTTTTTTCACGTCACGCGTCCATGGAGATCTTTGTCTGCAACTGAAAAG TTTATACCTTACCTGGAACAAATGGTTGAAACATACGAGGCTAATATCGGCTTATTAG GAATAGTCCCTGTACTAATAAAATCAGGTGGATCAGTTGATCAGTATATTTTGGACGA AGCTCGGAAAGAATTTGGAGATGACTTGCTTAATTCCACAATTAAATTAAGGGAAA GAATAAAGCGATTTGATGTTCAAGGAATCACGGAAGAAGATACTCATGATAAAGAA GCTCTAAAACTATTCAATAACCTTACAATGGAATTGATCGAAAGGGTGGAAGGTTAA TGGTACGAAAATTAGGGGATCTACCTAGAAAGCCACAAGGCGATAGGTCAAGCTTA AAGAACCCTTACATGGATCTTACAGATTCTGAAAGTAAAGAAACAACAGAGGTTAA ACAAACAGAACCAAAAAGAAAAAAAGCATTGTTGAAAACAATGAAAGTTGATGTT TCAATCCATAATAAGATTAAATCGCTGCACGAAATTCTGGCAGCATCCGAAGGGAAT TCATATTACTTAGAGGATACTATTGAGAGAGCTATTGATAAGATGGTTGAGACATTAC CTGAGAGCCAAAAAACTTTTTATGAATATGAATTAAAAAAAAGAACCAACAAAGGC TGAGACAGACTCCAAACGAGTCTGTTTTTTTAAAAAAAATATTAGGAGCATTGAATA TATATTAGAGAATTAAGAAAGACATGGGAATAAAAATATTTTAAATCCAGTAAAAATA TGATAAGATTATTTCAGAATATGAAGAACTCTGTTTGTTTTTGATGAAAAAACAAACA AAAAAAATCCACCTAACGGAATCTCAATTTAACTAACAGCGGCCAAACTGAGAAGT TAAATTTGAGAAGGGGAAAAGGCGGATTTATACTTGTATTTAACTATCTCCATTTTAA CATTTTATTAAACCCCATACAAGTGAAAATCCTCTTTTACACTGTTCCTTTAGGTGATC GCGGAGGGACATTATGAGTGAAGTAAACCTAAAAGGAAATACAGATGAATTAGTGT ATTATCGACAGCAAACCACTGGAAATAAAATCGCCAGGAAGAGAATCAAAAAAGG GAAAGAAGAAGTTTATTATGTTGCTGAAACGGAAGAGAAGATATGGACAGAAGAG

	CAAATAAAAAACTTTTCTTTAGACAAATTTGGTACGCATATACCTTACATAGAAGGTC ATTATACAATCTTAAATAATTACTTCTTTGATTTTTGGGGCTATTTTTTAGGTGCTGAA GGAATTGCGCTCTATGCTCACCTAACTCGTTATGCATACGGCAGCAAAGACTTTTGCT TTCCTAGTCTACAAACAATCGCTAAAAAAATGGACAAGACTCCTGTTACAGTTAGAG GCTACTTGAAACTGCTTGAAAGGTACGGTTTTATTTGGAAGGTAAACGTCCGTAATA AAACCAAGGATAACACAGAGGAATCCCCGATTTTTAAGATTAGACGTAAGGTTCCTT TGCTTTCAGAAGAACTTTTAAATGGAAACCCTAATATTGAAATTCCAGATGACGAGG AAGCACATGTAAAGAAGGCTTTAAAAAAGGAAAAAGAGGGTCTTCCAAAGGTTTT GAAAAAAGAGCACGATGAATTTGTTAAAAAAATGATGGATGAGTCAGAAACAATTA ATATTCCAGAGGCCTTACAATATGACACAATGTATGAAGATATACTCAGTAAAGGAGA AATTCGAAAAGAAATCAAAAAACAAATACCTAATCCTACAACATCTTTTGAGAGTATA TCAATGACAACTGAAGAGGAAAAAGTCGACAGTACTTTAAAAAGCGAAATGCAAA ATCGTGTCTCTAAGCCTTCTTTTGATACCTGGTTTAAAAACACTAAGATCAAAATTGA AAATAAAAATTGTTTATTACTTGTACCGAGTGAATTTGCATTTGAATGGATTAAGAAA AGATATTTAGAAACAATTAAAACAGTCCTTGAAGAAGCTGGATATGTTTTCGAAAAA ATCGAACTAAGAAAAGTGCAATAAACTGCTGAAGTATTTCAGCAGTTTTTTTTATTTA GAAATAGTGAAAAAAATATAATCAGGGAGGTATCAATATTTAATGAGTACTGATTTAA ATTTATTTAGACTGGAATTAATAATTAACACGTAGACTAATTAAAATTTAATGAGGGAT AAAGAGGATACAAAAATATTAATTTCAATCCCTATTAAATTTTAACAAGGGGGGGATT AAAATTTAATTAGAGGTTTATCCACAAGAAAAGACCCTAATAAAATTTTTACTAGGGT TATAACACTGATTAATTTCTTAATGGGGGAGGGATTAAAATTTAATGACAAAGAAAA CAATCTTTTAAGAAAAGCTTTTAAAAGATAATAATAAAAAGAGCTTTGCGATTAAGC AAAACTCTTTACTTTTTCATTGACATTATCAAATTCATCGATTTCAAATTGTTGTTGTAT CATAAAGTTAATTCTGTTTTGCACAACCTTTTCAGGAATATAAAACACATCTGAGGCT TGTTTTATAAACTCAGGGTCGCTAAAGTCAATGTAACGTAGCATATGATATGGTATAG CTTCCACCCAAGTTAGCCTTTCTGCTTCTTCTGAATGTTTTTCATATACTTCCATGGGT ATCTCTAAATGATTTTCCTCATGTAGCAAGGTATGAGCAAAAAGTTTATGGAATTGAT AGTTCCTCTCTTTTTCTTCAACTTTTTTATCTAAAACAAACACTTTAACATCTGAGTCA ATGTAAGCATAAGATGTTTTTCCAGTCATAATTTCAATCCCAAATCTTTTAGACAGAA ATTCTGGACGTAAATCTTTTGGTGAAAGAATTTTTTTATGTAGCAATATATCCGATACA GCACCTTCTAAAAGCGTTGGTGAATAGGGCATTTTACCTATCTCCTCTCATTTTGTGG AATAAAAATAGTCATATTCGTCCATCTACCTATCCTATTATCGAACAGTTGAACTTTTTA ATCAAGGATCAGTCCTTTTTTTCATTATTCTTAAACTGTGCTCTTAACTTTAACAACTC GATTTGTTTTTCCAGATCTCGAGGGTAACTAGCCTCGCCGATCCCGCAAGAGGCCCG GCAGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTT CTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAAT AATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTT TTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAA AGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACA GCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACT TTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAA CTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACA GAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACC ATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGA GCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGA ACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAG CAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCC GGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGC TCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGG GTC**G**CGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGT TATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTG AGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATAT ACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTT TTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGA

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pGGAselect*	gaattcTCGAGGCGGCCGCATGTGAGTCTCCCTATAGTGAGTCGTATTAATTTCGCGG GCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGT AGCACCAGGCGTTTAAGGGCACCAATAACTGCCTTAAAAAAATTACGCCCCGCCCT GCCACTCATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATC ACAAACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCG TATAATATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGCCACGT TTAAATCAAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACAAAAAACATATTCT CAATAAACCCTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCG AATATATGTGTAGAAACTGCCGGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAA ACGTTTCAGTTTGCTCATGGAAAACGGTGTAACAAGGGTGAACACTATCCCATATCA CCAGCTCACCGTCTTTCATTGCCATACGAAATTCCGGATGAGCATTCATCAGGCGGG CAAGAATGTGAATAAAGGCCGGATAAAACTTGTGCTTATTTTTCTTTACGGTCTTTAA AAAGGCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTG AAATGCCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCA GTGATTTTTTTCTCCATTTTAGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAA TACGCCCGGTAGTGATCTTATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGA TCAAAGTCTCATTTTCGCCAAAAGTTGTCATGACCAAAATCCCTTAACGTGAGTTTTC GTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTT TTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGT TTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAG AGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAA GAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCT GCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGA TAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGA GCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCA CGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAA CAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCC TGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGG GGCGGAGCCTATGGAAAAACGCCAGCAATGCGGCCTTTTTACGGTTCCTGGCCTTT TGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCG TATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCA GCGAGTCAGTGAGCGAGGAAGCCGAAAAATCAATAATCAGACAACAAGATGTGCG AACTCGATATTTTACACGACTCTCTTTACCAATTCTGCCCCGAATTACACTTAAAACG ACTCAACAGCTTAACGTTGGCTTGCCACGCATTACTTGACTGTAAAACTCTCACTCTT ACCGAACTTGGCCGTAACCTGCCAACCAAAGCGAGAACAAAACATAACATCAAACG AATCGACCGATTGTTAGGTAATCGTCACCTGCAGGAAGGTTTAAACGCATTTAGGTG ACACTATAGAAGTGTGTATCGCTCGAGggatcc

Table S3. Previously reported *Bacillus Cp*PGG expression systems.

P-SP	Backbone plasmid	Medium	Scale	Strain	Activation	Activity	Conclusion	Reference
PAmyL-GlmU	pHY	LB+2% lactose	Shake-flask	WB600	Host-mediated	0.16 U/ml	Most-effective SP after screening	(Niu et al. 2019)
PAmyL-GlmU-R	pHY	LB+2% lactose	Shake-flask	WB600	Host-mediated	0.23 U/ml	Most-effective after SP mutation	
PAmyL-GlmU-R	pHY	LB+40 g/L lactose	Fed-batch 25 L Bioreactor	*B. licheniformis* CBBD302	Host-mediated	6.8 U/ml	Final process	
P_{43}-NprB	pP43NMK	TB	Shake-flask	WB600	Host-mediated	1.5 U/ml	Baseline	(Yin et al. 2021)
PSecA-NprB	pP43NMK	TB	Shake-flask	WB600	Host-mediated	2.02 U/ml	Second most-effective, but selected for SP selection after PYvyD was ruled out	
PYvyD-NprB	pP43NMK	TB	Shake-flask	WB600	Host-mediated	2.21 U/ml	Most-effective P, but higher PGG activity compromised *Bacillus* transformation and growth ability	
PSecA-YdeJ	pP43NMK	TB	Shake-flask	WB600	Host-mediated	3.23 U/ml	Most-effective SP	
PSecA-GlmU	pP43NMK	TB	Shake-flask	WB600	Host-mediated	< 0.5 U/ml	Low activity	
PYvyD-YdeJ	pP43NMK	TB	Shake-flask	WB600	Host-mediated	< 1.25 U/ml	Low activity	
PSecA-YdeJ	pP43NMK	TB1	Fed-batch 5 L Bioreactor	WB600	Host-mediated	7.07 U/ml	Final process	
Pgrac-amyQ	pHT43	TB	Shake-flask	DB403	Host-mediated	0.5 U/ml	Self-activation in DB403	(Ouyang et al. 2021)
Pgrac-amyQ	pHT43	TB	Shake-flask	168	Host-mediated	0.6 U/ml	Self-activation in 168	
Pgrac-amyQ	pHT43	TB	Shake-flask	WB800N	Exogenous	Inactive	No self-activation in WB800N	
Pgrac-amyQ	pHT43	TB	Shake-flask	WB800N	Exogenous (trypsin)	2.77 U/ml	Baseline	(Zhang et al. 2024)
Pgrac-amyQ	pHTN07	TB	Shake-flask	WB800N	Exogenous (trypsin)	11.9 U/ml	Backbone modification significantly improved activity	
PSecA-YdeJ [1]	pP43NMK	TB	Shake-flask	168-derived	Host-mediated	5.25 U/ml	Baseline of A291S mutant in food-grade system	(Teng et al. 2024)
PSecA-YdeJ[1]	pP43NMK	TB	Fed-batch 5 L Bioreactor	168-derived	Host-mediated	8.15 U/ml	Highest value of A291S mutant in food-grade system	
PSecA-YdeJ	pP43NMK	TB	Fed-batch 5 L Bioreactor	168-derived	Host-mediated	6.06 U/ml	PSecA-YdeJ functionality in food-grade system	

[1] Expression of a mutant *Cp*PGG, not comparable with the above expression systems of wt *Cp*PGG.

Table S4. Other reported *Bacillus* CpPGG expression systems based on protease co-expression and intein-mediated activation.

P-SP	Backbone plasmid	Medium	Scale	Strain	Activation	Activity	Conclusion	Reference
PSecA-sfGFP	pHTN06	TB	Shake-flask	WB800N	Exogenous (trypsin)	< 3 U/ml	Low activity when combined with sfGFP	(Zhang et al. 2024)
Pgrac-sfGFP	pHTN06	TB	Shake-flask	WB800N	Exogenous (trypsin)	26 U/ml	sfGFP outperformed conventional SP	
P556-sfGFP	pHTN06	TB	Shake-flask	WB800N	Exogenous (trypsin)	36.9 U/ml	After promoter and RBS optimization	
PSecA-SP[1]	pMK	TB	Shake-flask	WB800-derived	AprE co-expression	8 U/ml	Baseline self-activating platform	(Teng et al. 2025)
Pmal160-SP[1]	pMK	Defined glucose-yeast extract	Fed-batch 5 L Bioreactor	WB800-derived	AprE co-expression	24.5 U/ml	Improvement after scale-up and promoter modification	
Pmal380-SP[1]	pMK	Defined glucose-yeast extract	Fed-batch 5 L Bioreactor	WB800-derived	AprE co-expression	53 U/ml	Highest self-activating mPGG platform	
PnprE-sfGFP	pP43NMK	Defined glucose-yeast extract	Fed-batch 5 L Bioreactor	WB600	Intein RIR1	23.6 U/ml	Protease-free strategy	(Geng et al. 2026)
PnprE-sfGFP	pP43NMK	Defined glucose-yeast extract	Fed-batch 5 L Bioreactor	WB600	Intein Mxe	37.2 U/ml	Mxe intein outperformed RIR1	

[1] Signal peptide not explicitly stated in Teng et al. (2025).

Figure S1. Evaluation of promoter-signal peptide combinations using mCherry as a secreted reporter. SDS-PAGE analysis (12%) of the extracellular fraction of *B. subtilis* 168 clones transformed with representative Reporter constructs. A prominent band corresponding to mCherry (~27 kDa) is observed for the Pgrac100-amyQ* construct, whereas only faint bands are detected for Pgrac100-GlmU* and PYvyD*-amyQ* combinations. M: molecular weight marker.

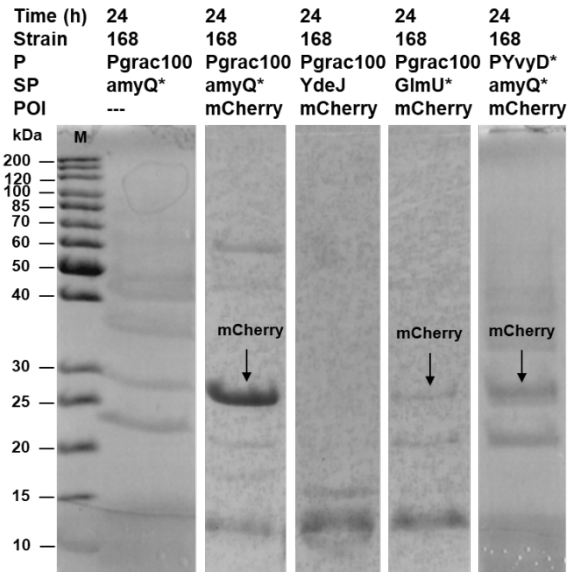


Figure S2. Visible reddish cell pellets obtained after centrifugation of *B. subtilis* 168 and KO7-S cultures carrying the Pgrac100-GlmU*-mCherry construct. The 168/pHT1469* was included as a negative control.

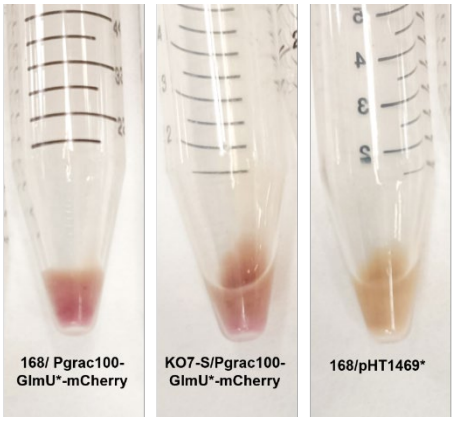


Figure S3. SDS-PAGE analysis (12%) of protein samples from batch Processes 1 and 2. The gel shows the 10-fold concentrated extracellular fractions collected at different time points. ProPGG and mPGG are indicated by a black arrow. The expected molecular weights are 35 kDa for ProPGG and 20 kDa for mPGG.

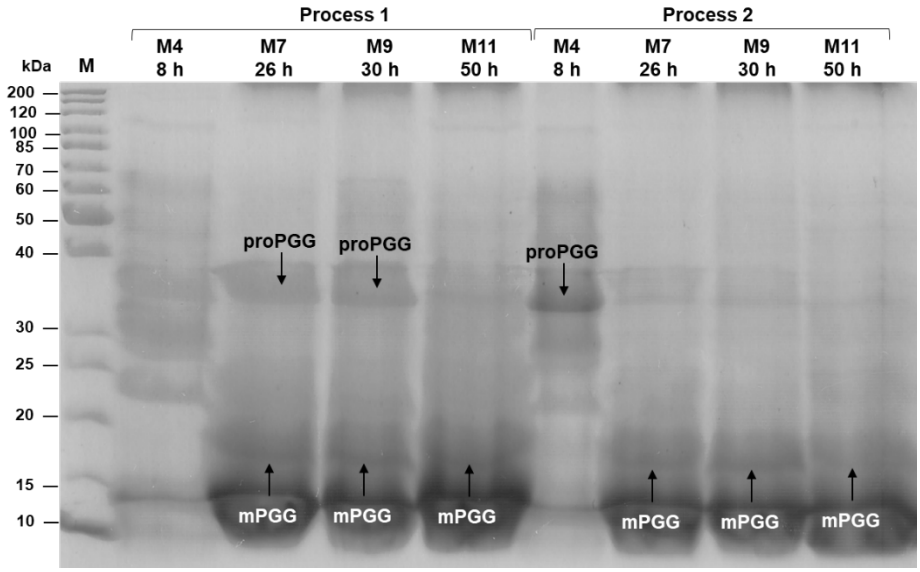


Figure S4. SDS-PAGE analysis (12%) of protein samples from fed-batch Processes 3 and 4. The gel shows the 10-fold concentrated extracellular fractions collected at different time points. No distinct bands corresponding to proPGG or mPGG were detected; instead, a diffuse low-molecular-weight protein pattern was observed in the extracellular fraction.

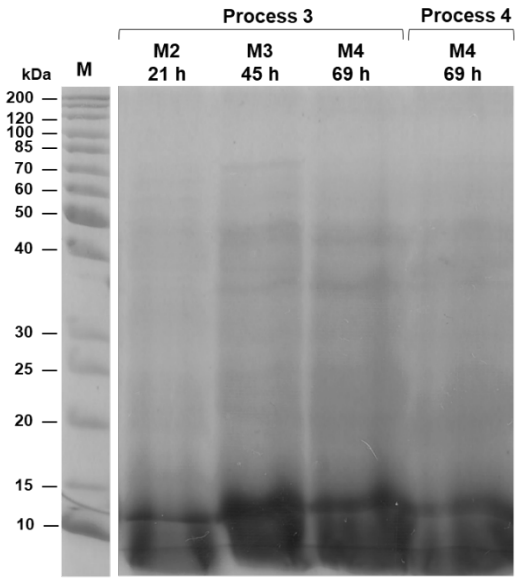
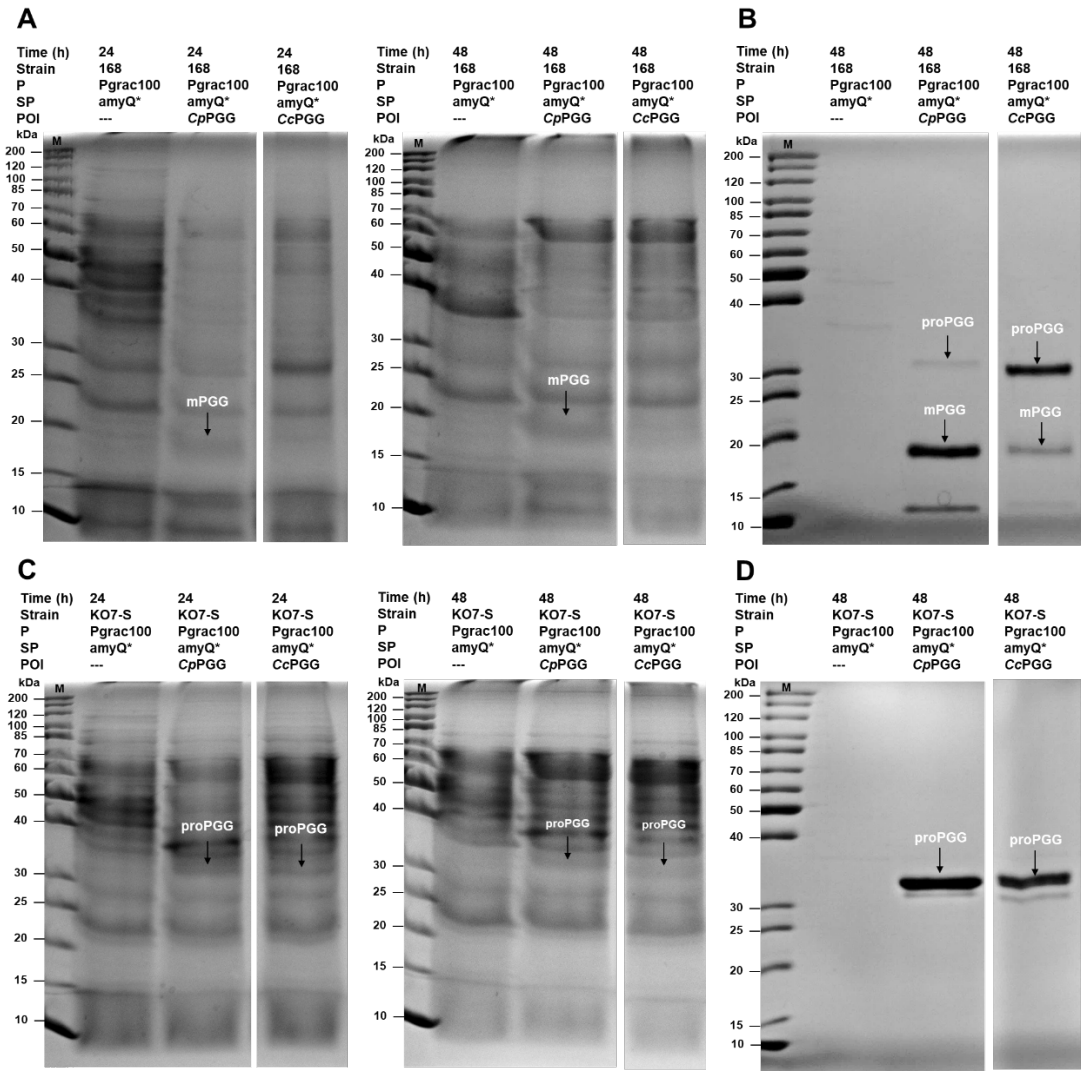


Figure S5. SDS-PAGE analysis (12%) of extracellular fractions obtained from *B. subtilis* 168 and the protease-deficient strain KO7-S expressing different PGG variants under the control of the Pgrac100-amyQ* secretion configuration. A. Extracellular fractions from *B. subtilis* 168 cultures collected at 24 h and 48 h post-induction. B. Purified proteins using HisTrap spin columns obtained from 48 h cultures of *B. subtilis* 168. *Cp*PGG is obtained predominantly as the mature form and the homolog *Cc*PGG mainly as the proenzyme. C. Extracellular fractions from KO7-S cultures collected at 24 h and 48 h post-induction. D. Purified proteins obtained from 48 h cultures of KO7-S, showing predominant accumulation of the proenzyme form for both constructs. Arrows indicate the expected molecular weights, proPGG, ~35 kDa, and mPGG, ~20 kDa. M: molecular weight marker.



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