Supplementary information

Programming biofilm-mediated multi-enzyme assembly cascade system for biocatalytic production of glucosamine from chitin

Jingjing Bao[1], Nian Liu[2], Liying Zhu[3], Qing Xu[4], He Huang[4], Ling Jiang[2,*]

[1]College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 210009, People's Republic of China; [2]College of Food Science and Light Industry, Nanjing Tech University, Nanjing 210009, People's Republic of China; [3]College of Chemical and Molecular Engineering, Nanjing Tech University, Nanjing 210009, People's Republic of China; [4]College of Pharmaceutical Sciences, Nanjing Tech University, Nanjing 210009, People's Republic of China

[*]Corresponding author: Ling Jiang, jiangling@njtech.edu.cn

Contents:

1) Construction of recombinant plasmids

The published coding sequences of *CsgA* (NCBI Reference Sequence: NC_000913.3) from *E. coli* K-12, *Tk-chiA* (GenBank accession: AB024740.1), *Tk-glmA* (GenBank accession: AB100422.1), *Tk-dac* (GenBank accession: AB125969.1) from *Thermococcus kodakaraensis* KOD1, *SpyTag* peptide with protein partner *SpyCatcher* (GenBank accession: JQ478411.1) and *SnoopTag* peptide with protein partner *SnoopCatcher* (GenBank accession: KU500646.1) from *Streptococcus pyogenes* and *Streptococcus pneumoniae* were optimized for expression in *E. coli* and synthesized respectively.

pET28a(+) CsgA-SpyTag (abr: pET-CsgA) was generated by fusing SpyTag to the C-terminus of *CsgA* using the primers CsgA-SpyTag_F and CsgA-SpyTag_R, after which the resulting fragment was digested with *Hind*III and *Xho*I and subcloned into pET-28a(+).

pET22b(+) SnoopCatcher-SpyCatcher (abr: pET-Catcher) was fully synthesized as a block protein, digested with *EcoR*I (F) and *Xho*I (R).

pET22b(+) SpyCatcher-glmA-SnoopTag (abr: pET-glmA) was generated as followed. Firstly, *SnoopTag* was fused to the C-terminus of *Tk-glmA* with the primers glmA-SnoopTag-F and glmA-SnoopTag-R, and the resulting fragment was digested with *Nde*I (F) and *Xho*I (R). Then, overlap-extension PCR was used to amplify *SpyCatcher* from pET-Catcher using the primers SpyCatcher-G-F and SpyCatcher-G-R, and the resulting fragment was digested with *Nde*I (F) and *Xho*I (R). The two resulting products were mixed and amplified again using the primers

SpyCatcher-G-F and glmA-SnoopTag-R.

pET22b(+) SpyTag-dac-SnoopTag (abr: pET-dac) was generated using the forward primer dac-F fused with *SpyTag* and the reverse primer dac-R fused with *SnoopTag*, and the resulting fragment was digested with *Nde*I (F) and *Xho*I (R).

pET22b(+) SpyTag-chiA-SnoopTag (abr: pET-chiA) was generated in the same way as pET22a SpyTag-dac-SnoopTag, using the primers chiA-F and chiA-R, digested with *Nde*I (F) and *Xho*I (R). A ~10 amino acid linker was added between enzymes and peptides or between two peptides.

2) Cloning, protein expression, purification and SDS-PAGE analysis

Unless stated otherwise, the recombinant fusion proteins were expressed and purified as followed. *E. coli* BL21 (DE3) cells (Vazyme Biotech Co. Ltd.) were transformed with recombined plasmids encoding the target proteins and an empty pET-22b(+) plasmid as a control group. Colonies were grown overnight at 37 °C with antibiotics and were then transferred into fresh lysogeny broth (LB) medium (50 mL) and grown at 37 °C until an OD_{600} of approximately 0.6. Enzyme expression was induced with 0.5 mM IPTG and continued for 8 h at 28 °C.

For the purification of proteins, cells were harvested by centrifugation and sonication for 10 min on ice in 50 mM potassium phosphate buffer (pH 7.0). Ni-NTA columns were used to purify proteins and ultrafiltration centrifuge tubes were used to concentrate proteins. The protein content was determined through the Bradford method, with bovine serum albumin as the standard. Purified proteins were analyzed

through SDS-PAGE. Especially, different purification method was carried out for CsgA-SpyTag. The cells from 50 mL fermentation broth was concentrated to 2 mL and supernatant was collected after sonication. 1% SDS and 250 mM sodium hydrate buffer was added to the supernatant, and the mixture was incubated for 1 h at room temperature. The mixture was then centrifuged for 30 min at 12,000 × g. The pellet was suspended in 5 mL of deionized water followed by another centrifugation. The pellet was dissolved in 100 μL of deionized water and freeze-dried for 12 h. We further added 50 μL of formic acid to obtain monomers of CsgA-SpyTag for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Solvents were removed by nitrogen flow.[1] The pellets were resuspended in 100 μL deionized water. **Table S3** showed the recovery of CsgA-SpyTag purification.

3) Quantitative Congo red binding assay

The Congo red (CR) binding assay was adapted from previously literature.[2] One mL from cultural broth expressing curli fibers was centrifuged at 5,000 × g for 15 min and resuspended in PBS with 0.03 mM Congo red dye. The mixture was incubated at 200 rpm and 25 ℃ for 20 min, after which the cells were collected at 12,000 × g for 15 min. The absorbance of the supernatant was measured under a full-wavelength scan in a SpectraMax M3 microplate reader (Molecular Devices Corporation, US). Congo red molecules bind strongly to fibrils on cells which express CsgA-SpyTag. Therefore, the absorption of Congo red solution was weakened after being incubated with fibrils on cell surface compared with the control group (**Figure S2a**). The amount of Congo red

dye bound by curli amyloids was calculated by the difference between the absorbance value of the control group (PBS + CR) and the experimental group. The correlation between the absorbance of supernatant and the amount of Congo red dye that was bound to curli fibers illustrated that fibers were gradually expressed when we increased the induction time of pET-CsgA (**Figure S2b**). Thus, the Congo red binding assay can be used to calculate the amount of curli fibers by the decrease in absorbance of the supernatant.

4) Thioflavin T (ThT) fluorescence assay

The ThT fluorescence assay was performed as reported previously,[2] using cell samples at appropriate dilution in 50 mM PBS buffer (pH 7.4) mixed with 5 μM ThT and incubated at 25 °C for 15 min. Samples (100 μL) were aliquoted into a 96-well plate. Fluorescence measurements were performed using a SpectraMax M3 microplate reader with excitation at 440 nm and a slit width of 5 nm. The fluorescence intensity of recombinant cells expressing CsgA-SpyTag biofilm increased significantly at 485 nm compared to the conrol strain (the *CsgA* deletion mutant PHL628) expressing no curli (**Figure S3**), which proved the existence of β-sheet structure in CsgA-SpyTag amyloid fibrils that extracellularly self-assembled and highly aggregated on cells surface.

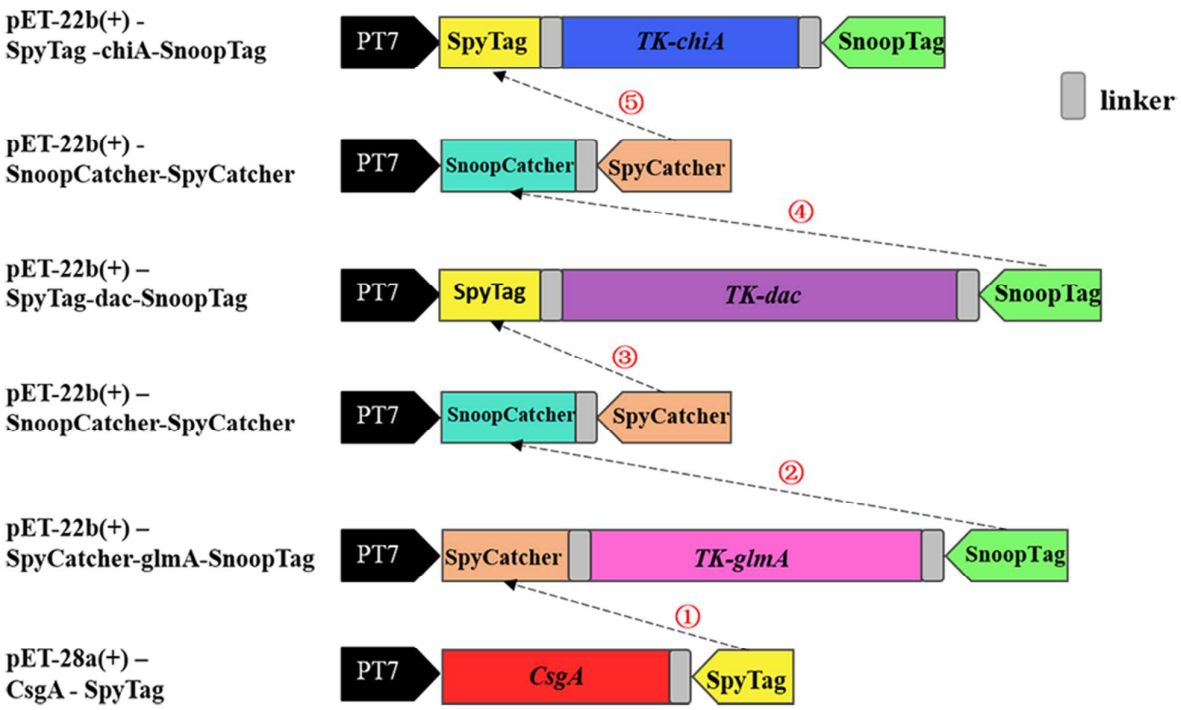

Figure S1. Construction of five recombinant plasmids.

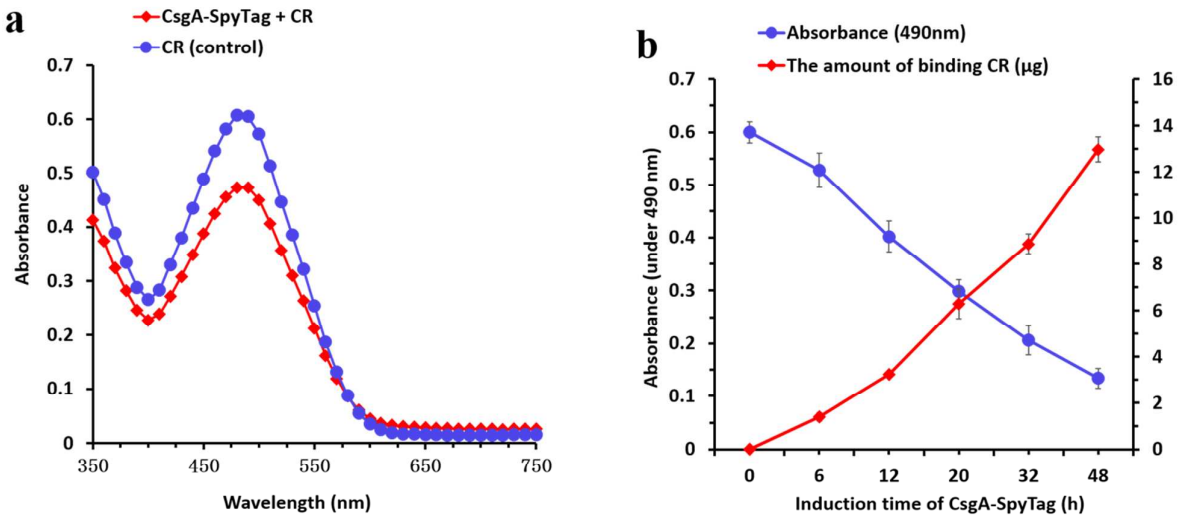

Figure S2. Congo red binding assay to test the existence of fibrils.

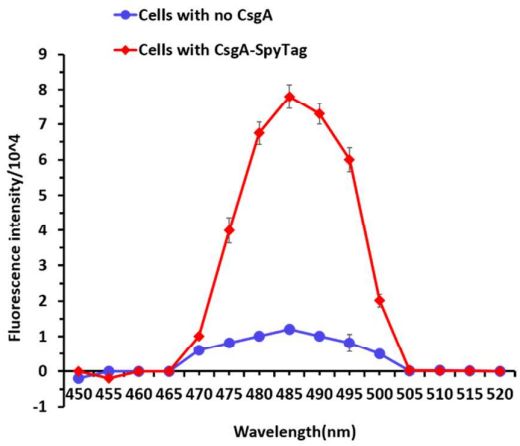

Figure S3. ThT binding fluorescence assay to test the existence of fibrils.

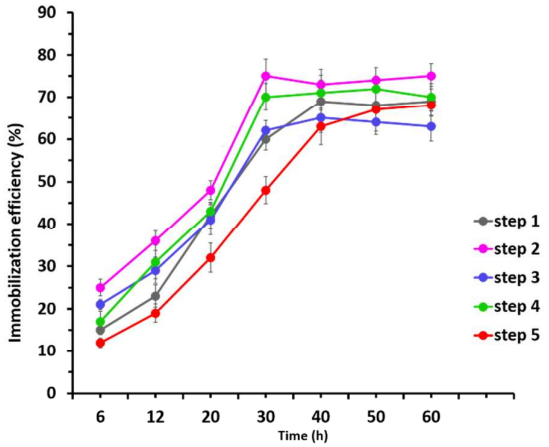

Figure S4. The linking efficiency of five steps in enzyme immobilization. The mole ratio of curli fibers to each step was 1: 1.5: 1.5: 1.5: 1.5: 1.5 in sequence.

The immobilization efficiency was calculated based on the following formula:

$$\eta\ (100\%) = \frac{m_t - m_i}{m_t} \times 100\% \qquad (1)$$

where m_t and m_i refers to the amount of the total enzyme protein added to the linking system initially and the amount of the residual enzyme protein that was recovered. To ensure effective bound between enzymes and biofilms, m_i contained two parts of free enzymes: enzymes collected from the supernatants of linking system after centrifugation and enzymes that were washed off from cell pellets, which was due to loose binding between counterparts and some non-covalent absorption of proteins. The washing buffer was 5 mL of 50 mM Na_2HPO_4-NaH_2PO_4.

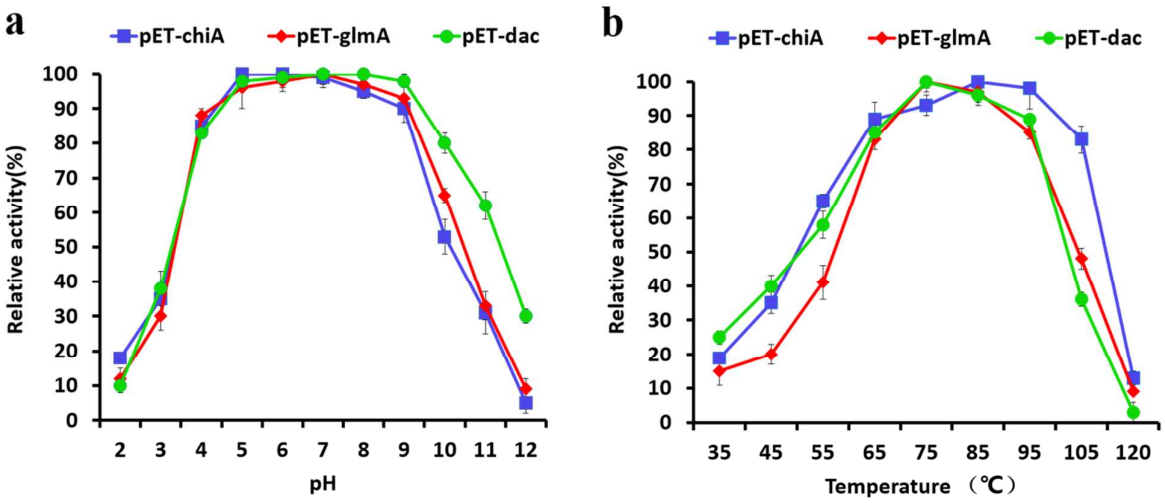

Figure S5. Relative activities of three enzymes under (a) pH ranging from 2–12 (b) temperature ranging from 35–120 °C. The maximum catalytic activity of each enzyme was defined as 100%.

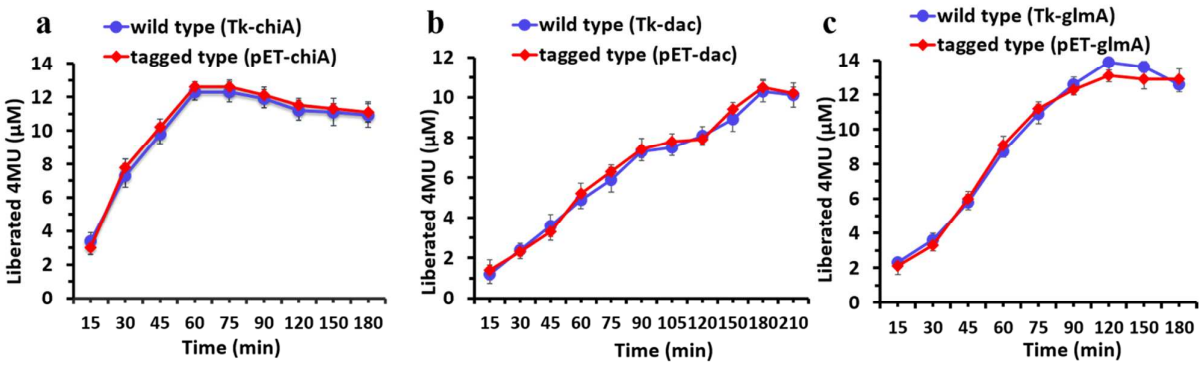

Figure S6. Comparison of wild type enzymes and tagged enzymes in product formation for (a) chiA, (b) dac, and (c) glmA. The reaction conditions was 75 °C and pH 7. The reaction mixture contained 25 μL of 1 mM $GlcNAc_3$-4MU and 50 ng of enzymes for enzyme chiA, 80 μL of 1 mM GlcNAc-4MU and 1 μg of enzymes for enzyme dac, and 25 μL of 1 mM 4-MUG mixed with 35 ng enzymes for enzyme glmA.

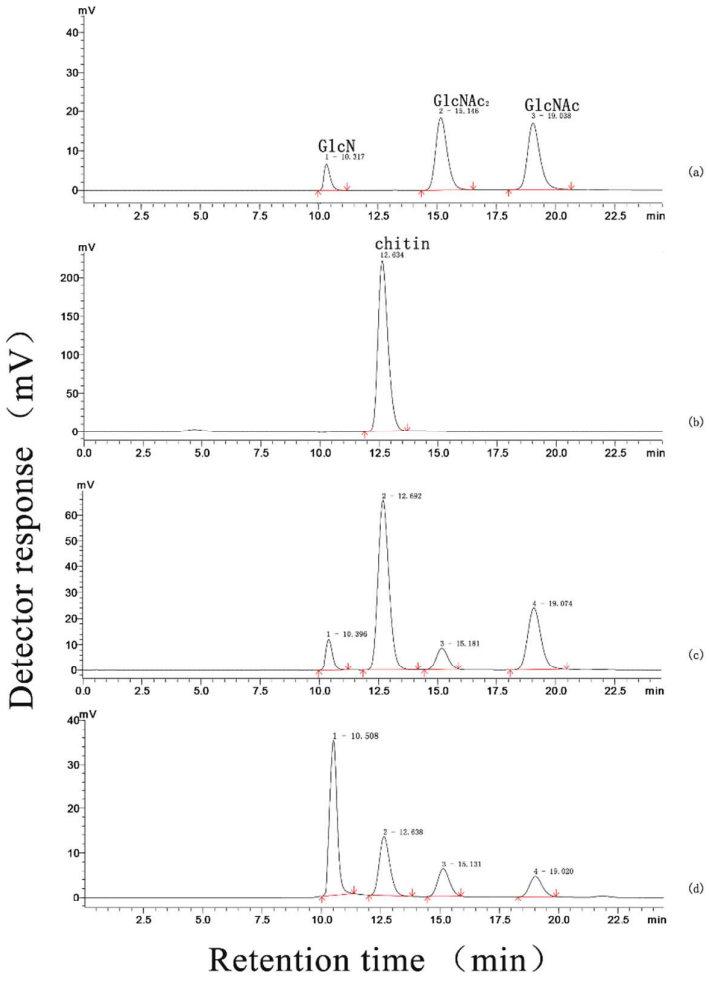

Figure S7. Analysis of products of the enzymatic reactions by HPLC. Samples were taken from the mixture of MAC system catalyzing α-chitin, which were shown in **Figure 4a, 4b**. (a) Standard GlcN, GlcNAc and $GlcNAc_2$ at a concentration of 1.5 mg/mL, 2.5 mg/mL, 2.5 mg/mL respectively; (b) 5.0% w/v of α-chitin; (c) reaction products of the original MAC system at 18 h; (d) reaction products of the modified MAC system at 18 h.

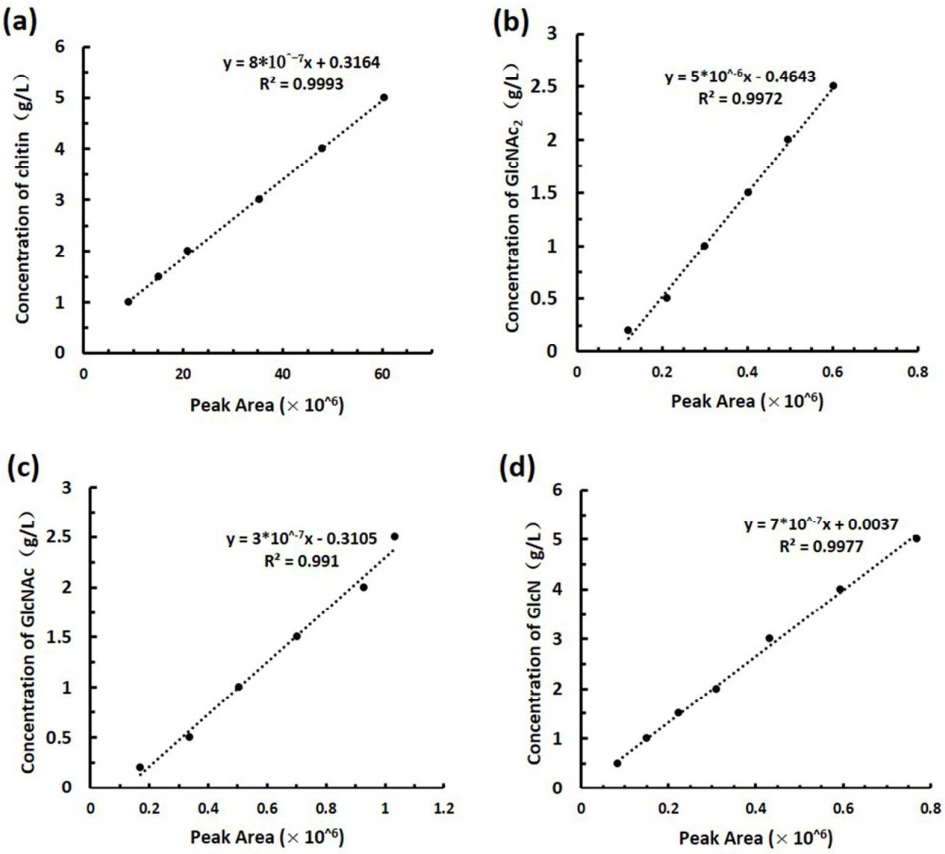

Figure S8. Standard curves of different substances relating to the catalysis process of chitin to GlcN by HPLC. Standard substances were diluted to different multiples to make sure that the concentrations of four samples fallen between the rang of the standard curves.

Table S1. *Escherichia coli* strains and plasmids used in this work

Strains or plasmids	Description/Phenotype	Reference/source
Strains		
DH5α	Host strain for general cloning	Vazyme
BL21(DE3)/pET28a-CsgA-SpyTag	The recombinant strain for protein expression of CsgA-SpyTag fusion protein	This study
BL21(DE3)/pET22b-SpyCatcher-glmA-SnoopTag	The recombinant strain for protein expression of SpyCatcher-glmA-SnoopTag fusion protein	This study
BL21(DE3)/pET22b-SnoopCatcher-SpyCatcher	The recombinant strain for protein expression of SnoopCatcher-SpyCatcher fusion protein	This study
BL21(DE3)/pET22b-SpyTag-dac-SnoopTag	The recombinant strain for protein expression of SpyTag-dac-SnoopTag fusion protein	This study
BL21(DE3)/pET22b-SpyTag-chiA-SnoopTag	The recombinant strain for protein expression of SpyTag-chiA-SnoopTag fusion protein	This study
PHL628	The csgA deletion mutant PHL628 as the control strain for expression no CsgA, MG1655 *malA-Kan ompR234* KanR Δ*csgA*	Previous study
Plasmid Name		
pET28a-CsgA-SpyTag	CsgA-SpyTag: Fusion of csgA to *Streptococcus pyogenes* split-adhesion tag. Under control of the T7 promoter. Kan.	This study (Nguyen et al., 2014)
pET22b-SpyTag-chiA-SnoopTag	SpyTag-chiA-SnoopTag: chiA isolated from *Thermococcus kodakaraensis* KOD1. N-terminally fused to the SpyTag domain and C-terminally fused to the SnoopTag domain. Under control of the T7 promoter. Amp.	This study (Tanaka, et al., 1999)
pET22b-SpyCatcher-glmA-SnoopTag	SpyCatcher-glmA-SnoopTag: glmA isolated from *Thermococcus kodakaraensis* KOD1. N-terminally fused to the SpyCatcher domain and C-terminally fused to the SnoopTag domain. Under control of the T7 promoter. Amp.	This study (Tanaka, et al., 2003)
pET22b-SnoopCatcher-SpyCatcher	Expression vector containing fusion protein of SnoopCatcher gene and SpyCatcher, with helical linker between them. Under control of the T7 promoter. Amp.	This study (Gianluca, et al., 2016)
pET22b-SpyTag-dac-SnoopTag	SpyTag-dac-SnoopTag: dac isolated from *Thermococcus kodakaraensis* KOD1.	This study

	N-terminally fused to the SpyTag domain and C-terminally fused to the SnoopTag domain. Under control of the T7 promoter. Amp.	(Tanaka, et al., 2004)

Table S2. Primers used in this work.

Primers name	Nucleotide Sequence
CsgA-SpyTag_F	5'-CCCAAGCTTATGAAACTTTTAAAAGTA
CsgA-SpyTag_R	5'-CCCTCGAG**TTATTTGGTGGGTTTATATGCATCGACCATAACGATGTGCGC**ACTGCCACCGCCACCGCTACCGCCACCGC
glmA-SnoopTag-F	GGGAATTCCATATGATGGGAAAGGTTGAGTTTAGCGGCA
glmA-SnoopTag-R	CCGCTCGAG*TTTGTTCACTTTAATAAATTCAATATCGCCCAGTTT*GGAGCCCCCGCCGGAGCCCCCGCCAAGAAGCTCAAC
SpyCatcher-G-F	GGGAATTCCATATGATGGGCAGCAGCCATCATCATCATCAT
SpyCatcher-G-R	CCGCTCGAGAATATGAGCGTCACCTTTAGTTGCTTTGCCA
dac-F	**GCGCACATCGTTATGGTCGATGCATATAAACCCACCAAATAA**GGCGGTGGCGGTAGCGGTGGCGGTGGCAGTCCATGGATC
dac-R	*TTTGTTCACTTTAATAAATTCAATATCGCCCAGTTT*GGAGCCCCCGCCGGAGCCCCCGCCCTCGAGCTTATTCACCTTAATA
chiA-F	**GCGCACATCGTTATGGTCGATGCATATAAACCCACCAAATAA**GGCGGTGGCGGTAGCGGTGGCGGTGGCAGTATGAAGAA
chiA-R	*TTTGTTCACTTTAATAAATTCAATATCGCCCAGTTTGGAGCCCCCGCCGGAGCCCCCGCC*CACCGGAACTGC

Restriction enzyme sites are underlined; SpyTag peptide sequence is in bold; SnoopTag peptide sequence is in italic; linker peptide sequence is double underlined.

Table S3. The preparation of CsgA-SpyTag fusion proeins [*].

Processing steps	Samples	Volume / mL	Amount of Congo red dye being bound [d] / mg	Recovery [f] /%
Step 1	Cell suspension [a]	50	ND [b]	ND
Step 2	Cell pellets [c]	2	0.68	100
Step 3	Purified CsgA-SpyTag	0.1	0.35	51.7

[*] Each experiment was carried out for three independent times.

[a] The cell suspension was from the 50 mL fermentation mixture.

[b] ND refers to not detected.

[c] Cells were collected by centrifugation and resuspended in 2 mL of 50 mM sodium acetate buffer (pH 7.0).

[d] Recovery of proteins equaled the ratio of purified proteins to that of cell pellets.

References.

(1) Wang, X.; Hammer, N. D.; Chapman, M. R. The molecular basis of functional bacterial amyloid polymerization and nucleation. *J. Biol. Chem.* **2008**, 283, 21530-21539.

(2) Nilsson, M. R. Techniques to study amyloid fibril formation in vitro. *Methods*. **2004**, 34, 151-160.