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Efficient production of extracellular pullulanase in *Bacillus subtilis* ATCC6051 using the host strain construction and promoter optimization expression system

Xin Liu[†], Hai Wang[†], Bin Wang and Li Pan^{*}

Abstract

Background: *Bacillus subtilis* has been widely used as a host for heterologous protein expression in food industry. *B. subtilis* ATCC6051 is an alternative expression host for the production of industrial enzymes, and exhibits favorable growth properties compared to *B. subtilis* 168. Extracellular expression of pullulanase from recombinant *B. subtilis* is still limited due to the issues on promoters of *B. subtilis* expression system. This study was undertaken to develop a new, high-level expression system in *B. subtilis* ATCC6051.

Results: To further optimize *B. subtilis* ATCC6051 as a expression host, eight extracellular proteases (*aprE*, *nprE*, *nprB*, *epr*, *mpr*, *bpr*, *vpr* and *wprA*), the sigma factor F (*spollAC*) and a surfactin (*srfAC*) were deleted, yielding the mutant *B. subtilis* ATCC6051Δ10. ATCC6051Δ10 showed rapid growth and produced much more extracellular protein compared to the wildtype strain ATCC6051, due to the inactivation of multiple proteases. Using this mutant as the host, eleven plasmids equipped with single promoters were constructed for recombinant expression of pullulanase (PUL) from *Bacillus naganoensis*. The plasmid containing the P_{spovG} promoter produced the highest extracellular PUL activity, which achieved 412.9 U/mL. Subsequently, sixteen dual-promoter plasmids were constructed and evaluated using this same method. The plasmid containing the dual promoter P_{amyL}-P_{spovG} produced the maximum extracellular PUL activity (625.5 U/mL) and showed the highest expression level (the dry cell weight of 18.7 g/L).

Conclusions: Taken together, we constructed an effective *B. subtilis* expression system by deleting multiple proteases and screening strong promoters. The dual-promoter P_{amyL}-P_{spovG} system was found to support superior expression of extracellular proteins in *B. subtilis* ATCC6051.

Keywords: *Bacillus subtilis*, Pullulanase, Protease deficient, Host construction, Promoter optimization

Background

Bacillus species have been widely used for the production of industrial enzymes [1]. *Bacillus* species are regarded as promising host strains with numerous advantages including: non-toxicity, convenience for gene modification and high yields of target proteins, fast growth rate and low

nutrient need [2]. Among *Bacillus* species, *Bacillus subtilis* is an attractive expression host due to its desirable features, such as having GRAS (generally recognized as safe) status, naturally efficient secretory system, the background knowledge is available concerning its genetics, physiology and large-scale fermentation processes [3, 4].

Recombinant expression is an important method to facilitate the production of target proteins. Many genetic strategies have been developed to improve the production of recombinant proteins, such as the use of protease-deficient host strains to prevent degradation [5], the deletion of extracellular protein genes to reduce secretion

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stress [6], and the optimization of promoters and signal peptides [7, 8]. Specifically, *B. subtilis* strains were engineered to serve as extracellular-protease-deficient strains for the overproduction of heterologous proteins such as *B. subtilis* WB600 [9], *B. subtilis* WB700 [10], and *B. subtilis* WB800 [11] (Table 1). These proteases-deficient strains were all constructed from the commonly used model and laboratory strain *B. subtilis* 168 [12]. *B. subtilis* 168 originates from the Marburg strain, which was deposited as *B. subtilis* ATCC 6051 (1930) and *B. subtilis* NCIB 3610 (1951), respectively [13]. Recently, *B. subtilis* ATCC6051 is an alternative expression host for production of industrial enzymes, which exhibits favorable growth properties as compared to the lab strain 168 [14]. Furthermore, gene expression systems of the genus *Bacillus* have been deeply investigated at the genome and transcriptome levels [15, 16]. In addition, the highly-active and controllable promoter is an important regulatory elements in expression systems. Recent research has focused on a novel and effective strategy for the identification of active promoters via screening of chromosomal DNA fragments and using a combinatory approach to construct two or more tandem promoters. Efficient expression vectors have been developed with different promoters like the Pr2 promoter of the sigW gene [17] and the pBL9 promoter of the glvA gene [18], the dual-promoter P_{gsiB}-P_{HpaII} system [19] and the P_{HpaII}-P_{amyQ}' system [20], etc.

Pullulanase (PUL, EC 3.2.1.41) is a debranching enzyme in the α -amylase family GH13, capable of specifically

hydrolyzing α -1,6-glycosidic linkages in pullulan, amylopectin, glycogen, and other related polysaccharides [35]. In food industries, there is a great commercial interest of using pullulanase, especially in the saccharification process together with saccharifying amylases for the production of high-glucose syrup and maltose [36]. Over the years, a large number of pullulanases have been discovered and identified from different microorganisms, and have been successfully heterologously expressed in *Escherichia coli* [37, 38] and various *Bacillus* species [35, 39]. However, only pullulanase derived from few strains such as *Bacillus acidopullulyticus* [40] and *Bacillus nagaensis* has great commercial value; and the production of pullulanase faces many difficulties, such as the expression levels of recombinant pullulanase were yet limit, low yields and low enzyme activity [36]. As shown in Table 1, the protease-deficient *B. subtilis* host strains (WB600, WB800) have been explored for overexpressing *B. nagaensis* pullulanase, but the expression levels are poorer, and cannot satisfy industrial needs. Therefore, the development of an efficient and easy-to-use expression system for the production of PUL is highly desirable.

It has been reported that *B. subtilis* ATCC 6051 could produce large amounts of foam, highly resistant spores, and multiple types of extracellular protease during fermentation [41–44]. In this study, to optimize *B. subtilis* ATCC6051 as an expression host, eight extracellular proteases (*aprE*, *nprE*, *nprB*, *epr*, *mpr*, *bpr*, *vpr* and *wprA*), the sigma factor F (*spoIIAC*) and a surfactin (*sfAC*) were deleted. We found that using this defective strain to

Table 1 Protein products from *B. subtilis*

Protein	Yield	Cultivation	Host	References
Alkaline β -mannanase	6041 U/mL	Shake flask	WB600	[21]
L-Asparaginase	407.6 U/mL	3 L bioreactor (fed-batch)	WB600	[22]
β -Glucanase	4840.4 U/mL	Shake flask	WB600	[23]
Alkaline α -amylase	196.35 U/mL	Shake flask	WB600	[24]
Pullulanase	11.7 U/mL	Shake flask	WB600	[25]
α -Acetolactate decarboxylase	135.8 U/mL	5 L fermenter	WB600	[26]
Catalase	8449 U/mL	Shake flask	WB600	[27]
Nattokinase	281 FU/mL	Shake flask	WB600	[28]
Penicillin G acylase	42 U/mL	Shake flask	WB600	[29]
Penicillin G acylase	0.26 U/mL	Shake flask	WB700	[30]
Staphylokinase	337 mg/L	2 L bioreactor (fed-batch)	WB700	[10]
Xylanase	6.93 U/mL	Shake flask	WB700	[31]
Xylanase	8.46 U/mL	Shake flask	WB800	[31]
Pullulanase	26.5 U/mL	Shake flask	WB800	[25]
Nattokinase	292 FU/mL	Shake flask	WB800	[28]
Endoxylanase	55 U/mL	Shake flask	WB800	[32]
α -Amylase	5566 U/mg	Shake flask	WB800	[33]
D-Mannose isomerase (Mlase)	51.2 U/mL	3 L fermenter	WB800	[34]

express heterologous protein production is superior to the common model laboratory strain. Furthermore, we constructed eleven single-promoter plasmids and sixteen dual-promoter plasmids for PUL expression in *B. subtilis* ATCC 6051Δ10. Taken together, we constructed an effective *B. subtilis* expression system by deleting multiple proteases and screening strong promoters, which has high potential for use in industrial applications.

Results

Construction of *B. subtilis* ATCC6051 as the heterologous expression host

In order to develop the wild type strain *B. subtilis* ATCC6051 as the alternative expression host with food-grade safety, we used the temperature-sensitive plasmid to inactivate eight protease genes, one spore-related gene and one surfactin gene, yielding the mutant ATCC6051Δ10 (Table 2 and Fig. 1a). Eight extracellular proteases (*aprE*, *nprE*, *nprB*, *epr*, *mpr*, *bpr*, *vpr*, *wprA*) were selected as the targets for deletion, because previous studies have demonstrated that the use of protease-deficient host strains could prevent protein degradation and reduce the secretory stress. As shown in Fig. 1b, the extracellular secreted proteins increased as more genes were deleted. A sigma factor F (*spoIIAC*) and a surfactin (*surfAC*) were also deleted, which affected the production of spores and foam during fermentation, respectively. During the shake flask fermentation process (48 h), *B. subtilis* ATCC6051 produced much foam and required to add 100 μl antifoam (25%, v/v) every 6 h; the mutant ATCC6051Δ10 produced much less foam at a controllable level that needed once antifoam at 24 h. After being cultured for 48 h in LB culture medium, the sporulation efficiency of ATCC6051 was 35.33% (122 colonies/300 colonies), while the sporulation efficiency of ATCC6051Δ10 was 0% (0 colonies/300 colonies),

which shows that ATCC6051Δ10 has great resistant to spore formation. Moreover, the mutant ATCC6051Δ10 achieved more extracellular protein production compared to that of ATCC6051 (Fig. 1c). And *B. subtilis* ATCC6051Δ10 (the dry cell weight of 14.7 g/L) grew faster than the wild-type stain ATCC6051 (Fig. 1d).

Additionally, the extracellular proteins increased as more genes were deleted, while some ever-present bands showed that there were still intense in ATCC6051Δ10. According to the molecular weights of these bands (Fig. 1b), corresponding proteases were predicted as: superoxide dismutase *sodA* (22.4 kDa), flagellin *hag* (32.6 kDa), RNA degradation presenting factor *YpfD* (42.3 kDa), dihydrolipoyl dehydrogenase *PdhD* (49.8 kDa) and wall-associated protein *WapA* (54.2 kDa). Without affecting the normal growth of the strain, we tried to inactivate a gene corresponding to the most obvious protein bands (*hag*, block mark, Fig. 1b), yielding the mutant ATCC6051Δ11: Δ10Δ *hag*. The extracellular protein became much more obvious, but protein backgrounds of this host strain became much more complicated (Fig. 1b). Therefore, the mutant ATCC6051Δ10 may be beneficial for the expression of target proteins, instead of ATCC6051Δ11: Δ10Δ*hag*.

Optimization of promoters for PUL expression in *B. subtilis* ATCC6051Δ10

Obtaining highly active promoters is an effective method for increasing the expression of heterologous proteins. The plasmids, which contained the PUL gene from *B. naganoensis* ATCC 53909, eleven promoters (Table 3, Eight promoters were from *B. subtilis*: P43, P_{spovG}, P_{aprE}, P_{amyE}, P_{hag}, P_{veg}, P_{nprE} and P_{nprB}; P_{amyL} and P_{glvA} were from *Bacillus licheniformis*; P_{sigW} was from *Bacillus amyloliquefaciens*) and the signal peptide of *amyQ* gene from *B. amyloliquefaciens* (SP_{amyQ}), were used to

Table 2 The deficient strains

Strains	Relevant properties	Genes	Gene description	Knockout length (bp)
ATCC6051Δ1	Δ <i>spoIIAC</i>	<i>spoIIAC</i>	The gene of RNA polymerase sigma-F factor	371
ATCC6051Δ2	Δ <i>spoIIAC</i> Δ <i>surfAC</i>	<i>surfAC</i>	The gene of surfactin synthase subunit	1024
ATCC6051Δ3	Δ <i>spoIIAC</i> Δ <i>surfAC</i> Δ <i>aprE</i>	<i>aprE</i>	The gene of serine protease AprE	189
ATCC6051Δ4	Δ <i>spoIIAC</i> Δ <i>surfAC</i> Δ <i>aprE</i> Δ <i>nprE</i>	<i>nprE</i>	The gene of bacillolysin	623
ATCC6051Δ5	Δ <i>spoIIAC</i> Δ <i>surfAC</i> Δ <i>aprE</i> Δ <i>nprE</i> Δ <i>nprB</i>	<i>nprB</i>	The gene of neutral protease B	297
ATCC6051Δ6	Δ <i>spoIIAC</i> Δ <i>surfAC</i> Δ <i>aprE</i> Δ <i>nprE</i> Δ <i>nprB</i> Δ <i>epr</i>	<i>epr</i>	The gene of minor extracellular protease Epr	326
ATCC6051Δ7	Δ <i>spoIIAC</i> Δ <i>surfAC</i> Δ <i>aprE</i> Δ <i>nprE</i> Δ <i>nprB</i> Δ <i>epr</i> Δ <i>mpr</i>	<i>mpr</i>	The gene of glutamyl endopeptidase Mpr	404
ATCC6051Δ8	Δ <i>spoIIAC</i> Δ <i>surfAC</i> Δ <i>aprE</i> Δ <i>nprE</i> Δ <i>nprB</i> Δ <i>epr</i> Δ <i>mpr</i> Δ <i>bpr</i>	<i>bpr</i>	The gene of bacillopeptidase F	1786
ATCC6051Δ9	Δ <i>spoIIAC</i> Δ <i>surfAC</i> Δ <i>aprE</i> Δ <i>nprE</i> Δ <i>nprB</i> Δ <i>epr</i> Δ <i>mpr</i> Δ <i>bpr</i> Δ <i>vpr</i>	<i>vpr</i>	The gene of extracellular serine protease Vpr	701
ATCC6051Δ10	Δ <i>spoIIAC</i> Δ <i>surfAC</i> Δ <i>aprE</i> Δ <i>nprE</i> Δ <i>nprB</i> Δ <i>epr</i> Δ <i>mpr</i> Δ <i>bpr</i> Δ <i>vpr</i> Δ <i>wprA</i>	<i>wprA</i>	The gene of cell wall-associated protease WprA	748
ATCC6051Δ11	Δ <i>spoIIAC</i> Δ <i>surfAC</i> Δ <i>aprE</i> Δ <i>nprE</i> Δ <i>nprB</i> Δ <i>epr</i> Δ <i>mpr</i> Δ <i>bpr</i> Δ <i>vpr</i> Δ <i>wprA</i> Δ <i>hag</i>	<i>hag</i>	The gene of flagellin	698

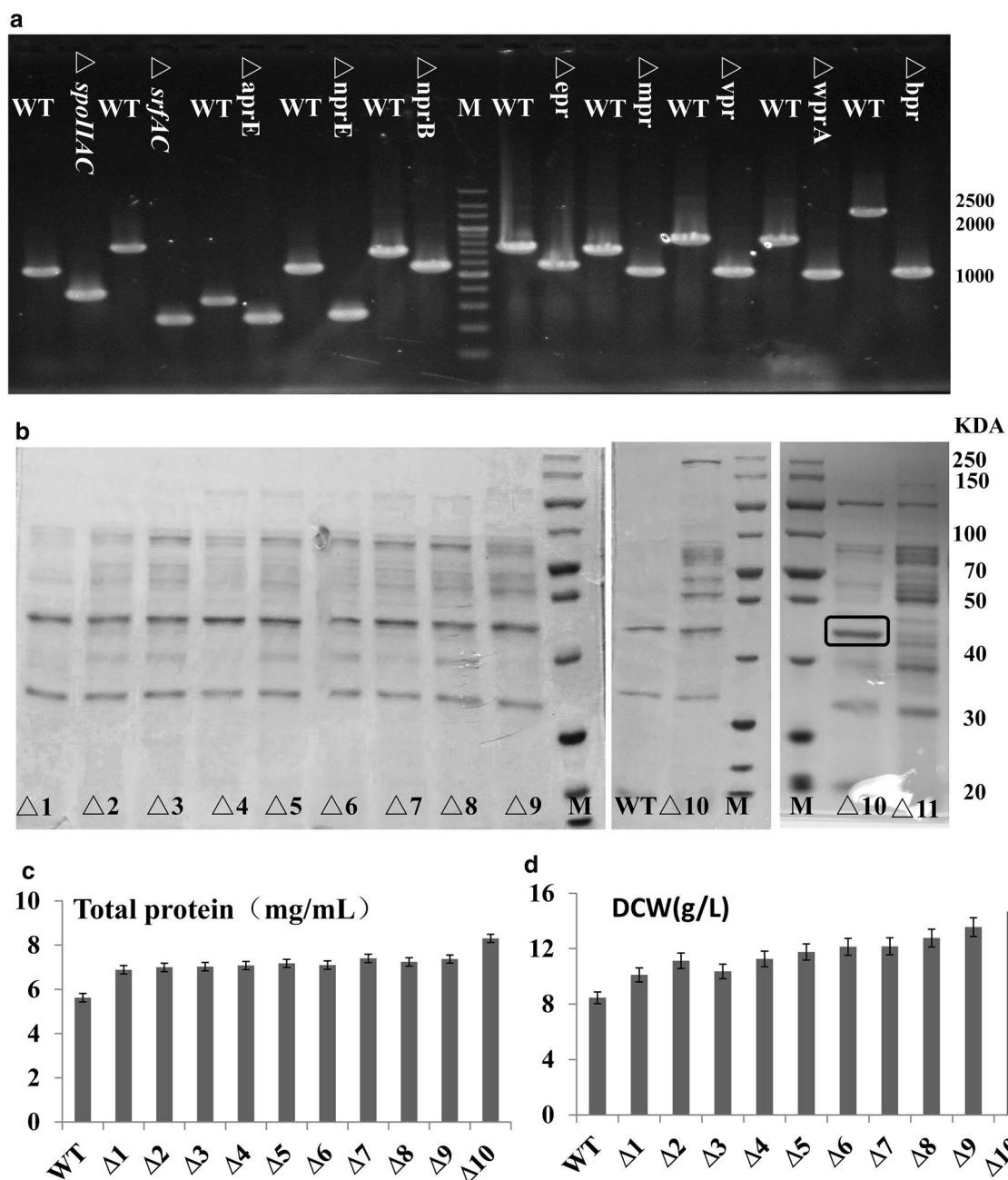


Fig. 1 The overview of *B. subtilis* ATCC6051 and the defective strains. **a** PCR verification of the ATCC6051 Δ 10 strain. Lane M, 200 bp DNA ladder. **b** SDS-PAGE analysis of ATCC6051 and eleven defective strains at 48 h. **c** The total protein of ATCC6051 and ten knockout strains at 48 h. **d** The cell growth of ATCC6051 and ten knockout strains at 48 h

construct the secretory expression with the plasmid pBE-MCS in the following: pBEPUL01, pBEPUL02, pBEPUL03, pBEPUL04, pBEPUL05, pBEPUL06, pBEPUL07, pBEPUL08, pBEPUL09, pBEPUL10 and pBEPUL11 (Table 4).

The eleven plasmids described above were used to transform *B. subtilis* ATCC6051 Δ 10 in which ten genes

were disrupted. The relative strengths of these promoters were determined by measuring the extracellular PUL activities of the eleven plasmid containing strains using orifice plate cultivation. As shown in Fig. 2a, the extracellular PUL activity of strains PUL01 through PUL11 were 72.5, 87.5, 5.2, 4.2, 5.1, 3.2, 1.8, 1.6, 25.7, 8.6 and 26.7 U/mL, respectively. Four (PUL01, PUL02, PUL09

Table 3 Properties of promoters used for PUL expression optimization

Promoter	Origin	Properties
P43	<i>B. subtilis</i>	Strong constitutive promoter [35]
P _{spovG}	<i>B. subtilis</i>	Promoter of septation protein spovG
P _{aprE}	<i>B. subtilis</i>	Promoter of alkaline protease [19]
P _{amyE}	<i>B. subtilis</i>	Promoter of α-amylase
P _{hag}	<i>B. subtilis</i>	Promoter of flagellin
P _{veg}	<i>B. subtilis</i>	Promoter of protein Veg
P _{nprE}	<i>B. subtilis</i>	Promoter of neutral protease E [19]
P _{nprB}	<i>B. subtilis</i>	Promoter of neutral protease B
P _{amyL}	<i>B. licheniformis</i>	Promoter of α-amylase, strong constitutive promoter [62]
P _{glvA}	<i>B. licheniformis</i>	Promoter of α-amylase [18]
P _{sigW}	<i>B. amyloliquefaciens</i>	Promoter of RNA polymerase sigma factor SigW [17]

Table 4 Strains and plasmids

Plasmid-containing strains	Plasmids	Promoter	Host
PUL0	pBE-MCS	None	<i>B. subtilis</i> ATCC6051Δ10
PUL1	pBEPUL01	P43	<i>B. subtilis</i> ATCC6051Δ10
PUL2	pBEPUL02	P _{spovG}	<i>B. subtilis</i> ATCC6051Δ10
PUL3	pBEPUL03	P _{aprE}	<i>B. subtilis</i> ATCC6051Δ10
PUL4	pBEPUL04	P _{amyE}	<i>B. subtilis</i> ATCC6051Δ10
PUL5	pBEPUL05	P _{hag}	<i>B. subtilis</i> ATCC6051Δ10
PUL6	pBEPUL06	P _{veg}	<i>B. subtilis</i> ATCC6051Δ10
PUL7	pBEPUL07	P _{nprE}	<i>B. subtilis</i> ATCC6051Δ10
PUL8	pBEPUL08	P _{nprB}	<i>B. subtilis</i> ATCC6051Δ10
PUL9	pBEPUL09	P _{amyL}	<i>B. subtilis</i> ATCC6051Δ10
PUL10	pBEPUL10	P _{glvA}	<i>B. subtilis</i> ATCC6051Δ10
PUL11	pBEPUL11	P _{sigW}	<i>B. subtilis</i> ATCC6051Δ10
PUL201	pBEPUL201	P _{spovG} -P _{spovG}	<i>B. subtilis</i> ATCC6051Δ10
PUL202	pBEPUL202	P _{spovG} -P43	<i>B. subtilis</i> ATCC6051Δ10
PUL203	pBEPUL203	P _{spovG} -P _{amyL}	<i>B. subtilis</i> ATCC6051Δ10
PUL204	pBEPUL204	P _{spovG} -P _{sigW}	<i>B. subtilis</i> ATCC6051Δ10
PUL205	pBEPUL205	P _{amyL} -P _{amyL}	<i>B. subtilis</i> ATCC6051Δ10
PUL206	pBEPUL206	P _{amyL} -P43	<i>B. subtilis</i> ATCC6051Δ10
PUL207	pBEPUL207	P _{amyL} -P _{spovG}	<i>B. subtilis</i> ATCC6051Δ10
PUL208	pBEPUL208	P _{amyL} -P _{sigW}	<i>B. subtilis</i> ATCC6051Δ10
PUL209	pBEPUL209	P43-P43	<i>B. subtilis</i> ATCC6051Δ10
PUL210	pBEPUL210	P43-P _{spovG}	<i>B. subtilis</i> ATCC6051Δ10
PUL211	pBEPUL211	P43-P _{amyL}	<i>B. subtilis</i> ATCC6051Δ10
PUL212	pBEPUL212	P43-P _{sigW}	<i>B. subtilis</i> ATCC6051Δ10
PUL213	pBEPUL213	P _{sigW} -P _{sigW}	<i>B. subtilis</i> ATCC6051Δ10
PUL214	pBEPUL214	P _{sigW} -P43	<i>B. subtilis</i> ATCC6051Δ10
PUL215	pBEPUL215	P _{sigW} -P _{spovG}	<i>B. subtilis</i> ATCC6051Δ10
PUL216	pBEPUL216	P _{sigW} -P _{amyL}	<i>B. subtilis</i> ATCC6051Δ10

and PUL11) of the 11 plasmid containing strains showed higher activity than others, and the PUL02 containing promoter P_{spovG} showed the highest PUL activity. Furthermore, the above four strains with high enzyme activity were expanded in shake-flask cultivation, the cell growth and PUL activities of these strains were compared. According to the results (Fig. 2b), the extracellular pullulanase activities were detected in different phases during the expression process, these four strains reached the stationary phase after 48 h. In the late phase of expression process, the maximum PUL activities were reached 412.9, 340.4, 301 and 159.5 U/mL under the control of promoter P_{spovG}, P_{amyL}, P43 and P_{sigW}, respectively. Meanwhile, the activity of PUL expressed by these four plasmid-containing strains indicated that the vitality effect among these four promoters were: P_{spovG}>P_{amyL}>P43>P_{sigW}. In addition, the dry cell weight of plasmid-containing strain PUL11 (P_{sigW}) showed the lowest expression level (9.32 g/L) and the plasmid-containing strain PUL02 (P_{spovG}) showed the highest expression level (14.76 g/L) at 48 h. As shown in Fig. 2c, SDS-PAGE analysis of supernatant proteins was carried out to verify these results, and the thicknesses of the appropriate bands (around 100 kDa) were in agreement with the PUL activity values.

PUL expression of dual promoters replacement in *B. subtilis* ATCC6051Δ10

Having compared eleven single promoters and identified promoters P_{spovG}, P_{amyL}, P43 and P_{sigW} as superior among them, with P_{spovG} being the strongest, dual-promoter plasmids were constructed to further increase PUL

expression. To create the dual promoter constructs, promoters P_{spovG} , P_{amyL} , P43 and P_{sigW} were combined with each other, yielding plasmids pBEPUL201, pBEPUL202, pBEPUL203, pBEPUL204, pBEPUL205, pBEPUL206, pBEPUL207, pBEPUL208, pBEPUL209, pBEPUL210, pBEPUL211, pBEPUL212, pBEPUL213, pBEPUL214, pBEPUL215 and pBEPUL216, respectively (Table 4). The sixteen dual-promoter plasmids were used to transform *B. subtilis* ATCC6051Δ10, yielding the sixteen corresponding plasmid-containing strains PUL201 through PUL216.

The expression strengths of the sixteen dual promoters were measured using shake-flask experiments similar to those used to assess the single promoters. As shown in Fig. 3a, the extracellular maximum PUL activities of plasmid-containing strains PUL201 through PUL216 during the expression process were 156.2 (P_{spovG} - P_{spovG}), 151.7 (P_{spovG} -P43), 77.1 (P_{spovG} - P_{amyL}), 582.9 (P_{spovG} - P_{sigW}), 358.1 (P_{amyL} - P_{amyL}), 556.2 (P_{amyL} -P43), 625.5 (P_{amyL} - P_{spovG}), 210.8 (P_{amyL} - P_{sigW}), 133.6 (P43-P43), 84.5 (P43- P_{spovG}), 141 (P43- P_{amyL}), 293.5 (P43- P_{sigW}), 25.44 (P_{sigW} - P_{sigW}), 119.7 (P_{sigW} -P43), 53 (P_{sigW} - P_{spovG}) and 48.3 (P_{sigW} - P_{amyL}) U/mL, respectively. Obviously, the plasmid-containing strain PUL207, which harbored the plasmid pBEPUL207 with the dual promoter P_{amyL} - P_{spovG} showed the highest PUL activity. This activity was almost 1.49-fold and 1.84 the activity produced by plasmid-containing strain PUL02 and PUL09, which expressed PUL using the P_{amyL} and P_{spovG} promoter, respectively. Two more plasmid-containing strains PUL204 (P_{spovG} - P_{sigW}) and PUL206 (P_{amyL} -P43) also have higher enzyme activity than the single promoter. Moreover, The dry cell weights of the sixteen plasmid-containing strains peaked at 36 h, and decreased substantially from 48 to 72 h. In particular, the plasmid-containing strain PUL207 (18.73 g/L) was much higher than that of the other fifteen plasmid-containing strains (Fig. 3b). SDS-PAGE analysis of the supernatant of the culture broth also indicated a distinctive protein band at around 100 kDa for the positive recombinant but not the negative control (Fig. 3c), consistent with the results of activity assay.

Discussion

Bacillus subtilis species are usually used as the host strains for heterologous protein expression. However, the host strains can produce many different types of extracellular protease which might degrade the target protein

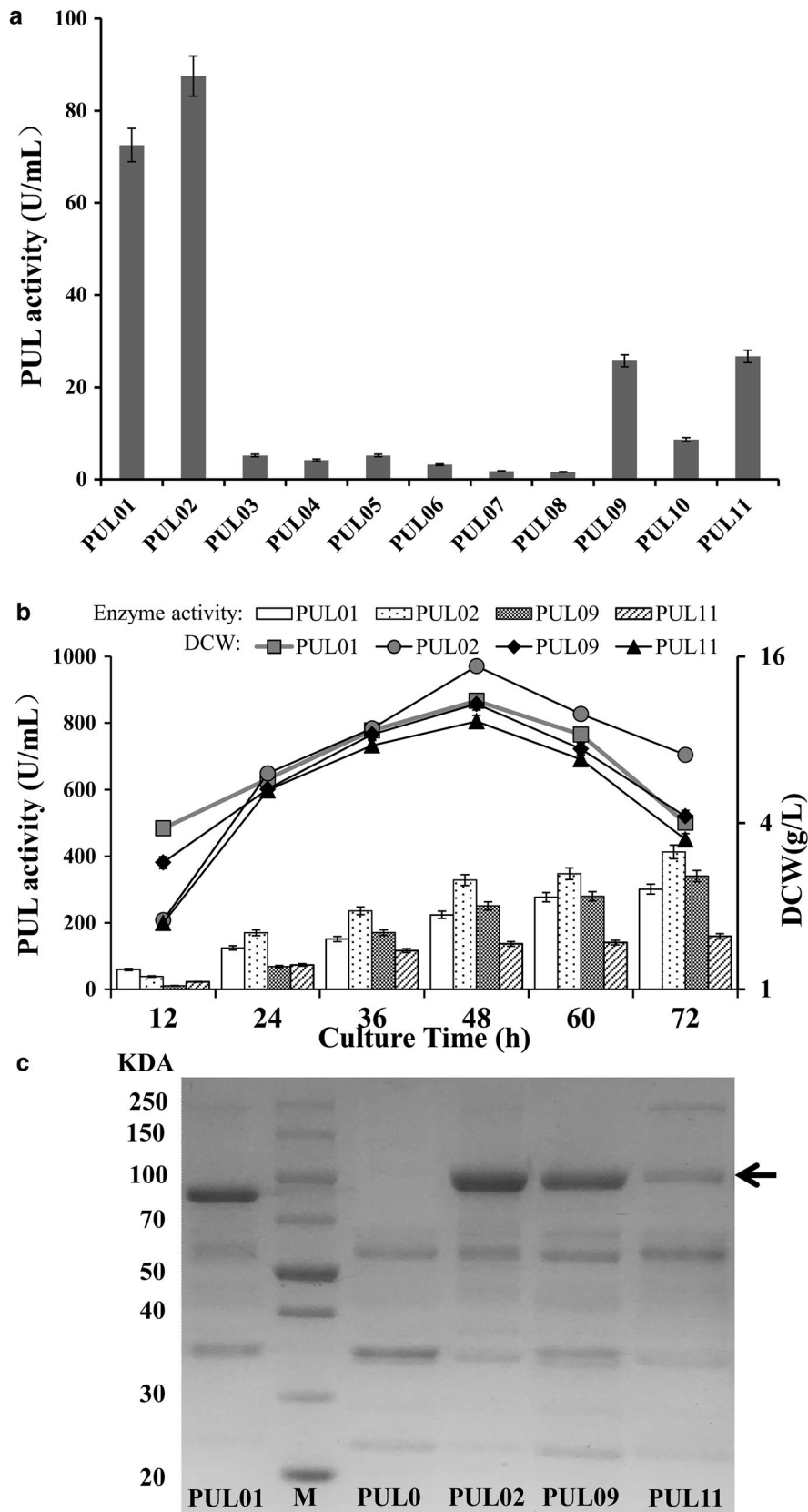
[1], and many protease-deficient strains display favorable heterologous protein production [43–45]. The multiple proteases-deficient host strains have been constructed, such as *B. subtilis* WB600, WB700 and WB800 [5, 46]. And the deficiency of proteases genes (*nprE*, *nprB*, *mpr*, *vpr*, *epi*, *bpr*, *wprA*, *aprE*) in *B. subtilis* improved the expression of target proteins obviously. Furthermore, Sigma F, encoded by *SpoIIAC* gene, controls the formation of forespore [47]. Gutierrez et al. showed that inactivation of sigma F could largely block the sporulation of *B. subtilis* [48]. Moreover, *B. subtilis* produces surfactin, an amphiphilic molecule which could accumulate at gas-liquid interfaces and induce foam during fermentation [49, 50]. *B. subtilis* ATCC 6051 produces a large amount of foam during fermentation, which has an extreme effect on fermentation process control and may lead to contamination [51]. This shows that surfactin may be a major mediator for foam formation, which may affect the protein secretion. In this study, eight extracellular proteases genes (*nprE*, *nprB*, *mpr*, *vpr*, *epi*, *bpr*, *wprA* and *aprE*), the sigma factor F gene (*SpoIIAC*) and the surfactin gene (*srfAC*) were knocked out to obtain the mutant ATCC 6051Δ10.

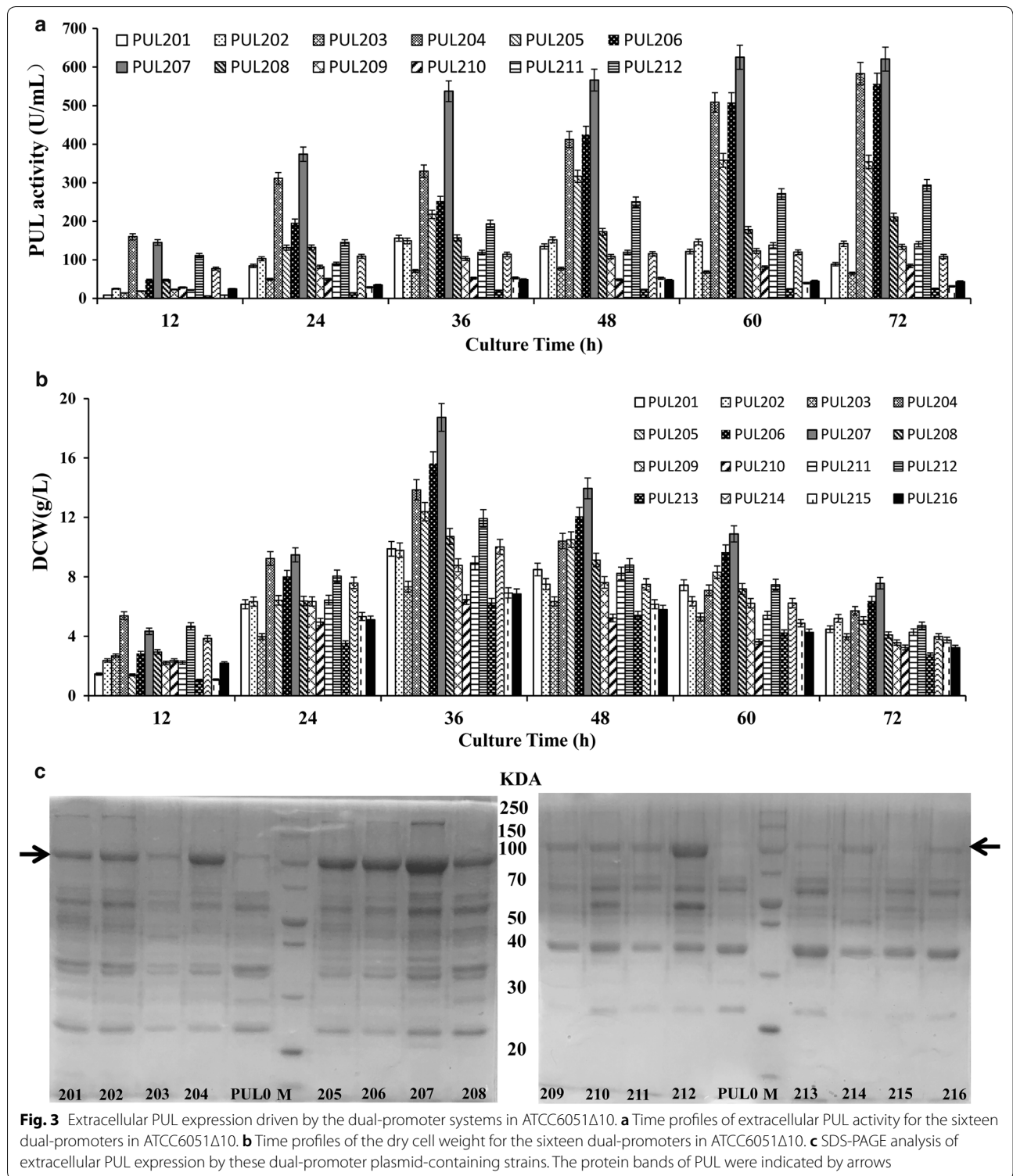
Due to the great demand of industrial applications, the overexpression of pullulanase has received extensive attention in recent years [52]. Among the effective solutions and prerequisites are the selection of new bacterial strains or the improvement of bacterial strains [53] and *B. subtilis* strains are well known for their ability to secrete a large number of useful extracellular enzymes [54]. In previous reports, several protease-deficient *B. subtilis* host strains have been explored for overexpressing *B. naganoensis* pullulanase, including WB600 [28], WB800 [22], and *B. subtilis* CCTCC M 2016536 (*srfC*, *spoIIAC*, *nprE*, *aprE* and *amyE* genes deletion) [20]. But these hosts showed poor expression of pullulanase and the extracellular activities reached 90.7 U/mL, which was the highest ever reported [20]. Secretory expression of PUL was achieved in ten-genes deficient *B. subtilis* ATCC 6051Δ10, and deletion of these ten genes indeed enhanced the production of PUL. This study provided a novel *B. subtilis* host, which has the potential for the heterologous expression of different proteins.

Obviously, promoter as an important regulatory element directly influences the expression efficiency of heterologous protein and strong promoters are usually used to achieve gene high-level expression [20]. In recent

(See figure on next page.)

Fig. 2 Extracellular PUL expression driven by the single-promoter systems in ATCC6051Δ10. **a** PUL activity of eleven single promoters in in well plates. **b** Time profiles of extracellular PUL activity and dry cell weight for the four high viability promoters in ATCC6051Δ10. **c** SDS-PAGE analysis of extracellular PUL expression by these single-promoter plasmid-containing strains. The protein bands of PUL were indicated by arrows





years, some previous reports have shown that many promoters in *B. subtilis* have been used for the expression of *B. naganensis* pullulanase. As known, Wang et al. [55] used three widely promoters (P_{43} , P_{apr} and P_{amy}) to

select the best promoter for PUL expression in *B. subtilis*, and the result indicated that P_{apr} (2.82 U/mL) is the most suitable for the production of PUL. Song et al. [35] constructed plasmids under the promoter P_{HpaII} and P_{43} ,

resulting in the extracellular PUL activity of 24.5 U/mL (P43). Other studies have shown that two or more tandem promoters can significantly increase the level of heterologous protein expression. Zhang et al. [20] showed that the tandem promoter P_{HpaII} - P_{amyQ} (90.7 U/mL) were found to elevate PUL productivity by 1.49 fold compared to the single P_{amyQ} (60.9 U/mL) system. Although the above promoters are used as strong promoters in *B. subtilis*, these promoters resulted in various levels of expression of the PUL reporter protein. In our work, a set of *B. subtilis* recombinants involving different combination of various promoters were constructed for heterologous expression of PUL from *B. naganensis*. Compared with the previous reports, the promoters P_{spovG} (412.9 U/mL), P_{amyL} (340.4 U/mL), and P43 (301 U/mL) can significantly increase the activity of PUL. Moreover, the dual promoters P_{amyL} - P_{spovG} (625.5 U/mL), P_{spovG} - P_{sigW} (582.9 U/mL) and P_{amyL} -P43 (556.2 U/mL) showed the higher extracellular PUL activity than the single promoters. Thus, the above promoter expression systems may have the potential for the production of useful proteins in *B. subtilis*.

Conclusion

In this study, the ten-genes-deficient strain *B. subtilis* ATCC6051 Δ 10 was obtained by overlap gene knockout. Compared with *B. subtilis* ATCC 6051, the mutant ATCC6051 Δ 10 had less foam during fermentation under the same conditions, displayed greater resistant to spore formation, achieved 1.48 times more extracellular protein production and 1.73 times more dry cell weight. Then, eleven single-promoter plasmids and sixteen dual-promoter plasmids were constructed and evaluated using shake-flask cultivation. By host strain construction and promoters optimization, the maximum PUL activity of 625.5 U/mL was achieved under the control of the dual promoter P_{amyL} - P_{spovG} . This study provides a valuable expression system with the potential application for industrial production of PUL, as well as expression of other proteins.

Materials and methods

Strains, plasmids and primers

The detailed information for genes, strains and plasmids were shown in Additional file 1: Table S1, and primers sequences were listed in Additional file 1: Table S2. The following concentrations of antibiotics were used for selection: 100 μ g/mL ampicillin (Amp), 5 μ g/mL erythromycin (Erm) and 20 μ g/mL kanamycin (Kan). Luria-Bertani (LB) medium consisted of 1% tryptone, 0.5% yeast extract, and 1% NaCl, with the pH 7. The flask fermentation medium contained LB medium with 1% corn starch.

Gene prediction

All the annotated gene sequences of the proteases from *B. subtilis* were selected from the NCBI database (<https://www.ncbi.nlm.nih.gov/>), and were used to find the corresponding genes in *B. subtilis* ATCC6051 by Blast tool (<http://blast.ncbi.nlm.nih.gov>).

Construction of the knockout vectors

The knockout vectors were constructed by a previously reported method [56], using the *mpr* gene as an example (pKS2-*mpr*, Additional file 1: Figure S1). The upstream and downstream homologous arms (about 500 bp) of *mpr* gene, named *mpr* (L) and *mpr* (R), were amplified using the primers of F-*mpr*-1/R-*mpr*-1 and F-*mpr*-2/R-*mpr*-2 (Additional file 1: Table S2) respectively, and the amplified fragments were further purified and recovered. By splicing overlap extension PCR method using the primers of F-*mpr*-1/R-*mpr*-2, the *mpr* (L) and *mpr* (R) were fused to form the fragment *mpr* (L+R), which was then cloned into the pKS2 (5094 bp) at the *EcoR* V restriction site. Other knockout vectors were constructed following the same method.

Gene knockout

The competent cells of *B. subtilis* were prepared by the method reported [57]. The competent cells were electro-transformed at 2.5 kV with 10 μ L knockout plasmid solution (100 ng/ μ L), maintained in 900 μ L recovery medium (LB medium added with 1% sorbitol) at 30 °C and 100 rpm for 4–6 h. The cells were spread onto LB plate with 5 μ g/mL erythromycin, incubated at 30 °C for 16–24 h, and the transformants were selected by PCR and plasmid extraction verification. The positive transformants were inoculated into LB liquid medium containing 5 μ g/mL erythromycin, cultured at 37 °C for 12 h and plated onto erythromycin LB solid medium, which was further incubated at 37 °C to screen the single-cross-over colonies. After PCR verification, the positive clones were subcultured in LB liquid medium without erythromycin at 30 °C for 12–16 h. At last, the cultures were incubated in LB plates at 37 °C for 20 h, and each single colony was inoculated onto a LB plate with erythromycin, as well as one without erythromycin. If a colony grew on the LB plate without erythromycin and was inhibited on LB plate with erythromycin, this strain could be the double crossover one, which would be further verified by PCR.

Construction of the PUL expression vector

The PUL expression vector was constructed based on the *pul* gene from *B. naganensis* ATCC 53909, the signal peptide of *amyQ* gene from *B. amyloliquefaciens* (SP_{*amyQ*}), the

P43 promoter and the terminator of *amyE* gene (T_{amyE}) from *B. subtilis* 168. First, the fragments of P43, SP_{amyQ} , *pul* and T_{amyE} were amplified by the primers (Additional file 1: Table S2) and purified, respectively. Second, the purified fragments of P43, SP_{amyQ} , *pul* gene and were fused by PCR to obtain the P43SPT fragment. Finally, the P43SPT fragment was inserted into the pBE-MCS plasmid at *EcoRI* and *XbaI* sites, which was then transformed into *Escherichia coli* HST08 to obtain the expression plasmid pBEPPUL01 (Additional file 1: Figure S2). Using the In-Fusion HD Cloning Plus kit [58], plasmid fragment one was joined with promoter fragments P_{spovG} , P_{aprE} , P_{amyE} , P_{hag} , P_{veg} , P_{nprE} , P_{nprB} , P_{amyL} , P_{glvA} , P_{sigW} and sixteen dual-promoter fragments to yield plasmids pBEPUL2 to pBEPUL11 and pBEPUL201 to pBEPUL216, respectively.

Analysis methods

According to a previously described method [59], the activity of PUL was assayed by the concentration of reducing sugars liberated into the reaction mixture and one unit of pullulanase was defined as the amount of enzyme required to produce the reducing sugar equivalent to 1 L/mol glucose per min. Data represent the average of three independent experiments, and error bars indicate the standard error of the mean value.

The cell growth was evaluated by measuring the dry cell weight (DCW) [60]. DNA and the proteins were determined by agarose gel electrophoresis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [61], respectively.

Additional file

Additional file 1: Figure S1. Construction procedure of the knockout vector (pKS2-mpr). All knockout vectors were constructed as shown. **Figure S2.** Construction procedure of the promoter plasmid (pBEPUL01). All expression plasmids were constructed as shown. **Table S1.** Genes, bacterial strains and plasmids used in this study. **Table S2.** primers used in this study.

Abbreviations

LB: Luria–Bertani; Amp: ampicillin; Kan: kanamycin; Erm: erythromycin; PCR: polymerase chain reaction; PUL: pullulanase; *spolAC*: the gene of RNA polymerase sigma-F factor; *srfAC*: the gene of surfactin synthase subunit; *aprE*: the gene of subtilisin E; *nprE*: the gene of bacillolysin; *nprB*: the gene of neutral protease B; *epr*: the gene of minor extracellular protease Epr; *mpr*: the gene of glutamyl endopeptidase Mpr; *vpr*: the gene of extracellular serine protease Vpr; *bpr*: the gene of bacillopeptidase F; *wprA*: the gene of cell wall-associated protease WprA; *hag*: the gene of flagellin; *amyE*: the gene of α -amylase; *amyQ*: the gene of α -amylase from *B. amyloliquefaciens*; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Authors' contributions

XL led the design and performance of the experiments, analysis of the data and writing of the paper. HW and LP participated in experimental design and

analysis. BW participated in editing the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions of this article are included in the manuscript and additional file.

Consent for publication

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Ethics approval and consent to participate

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References

- Westers L, Westers H, Quax WJ. *Bacillus subtilis* as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. *BBA-Mol Cell Res*. 2004;1694(1–3):299–310.
- van Dijk JM, Hecker M. *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. *Microb Cell Fact*. 2013. <https://doi.org/10.1186/1475-2859-12-3>.
- Schallmey M, Singh A, Ward OP. Developments in the use of *Bacillus* species for industrial production. *Can J Microbiol*. 2004;50(1):1–17.
- Zhang X, Zhang YP. One-step production of biocommodities from lignocellulosic biomass by recombinant cellulolytic *Bacillus subtilis*: opportunities and challenges. *Eng Life Sci*. 2010;10(5):398–406.
- Durban MA, Silbersack J, Schweder T, Schauer F, Bornscheuer UT. High level expression of a recombinant phospholipase C from *Bacillus cereus* in *Bacillus subtilis*. *Appl Microbiol Biotechnol*. 2007;74(3):634–9.
- Waldeck J, Meyer-Rammes H, Wieland S, Feesche J, Maurer K, Meinhardt F. Targeted deletion of genes encoding extracellular enzymes in *Bacillus licheniformis* and the impact on the secretion capability. *J Biotechnol*. 2007;130(2):124–32.
- Degering C, Eggert T, Puls M, Bongaerts J, Evers S, Maurer K, Jaeger K. Optimization of protease secretion in *Bacillus subtilis* and *Bacillus licheniformis* by screening of homologous and heterologous signal peptides. *Appl Environ Microb*. 2010;76(19):6370–6.
- Samant S, Gupta G, Karthikeyan S, Haq SF, Nair A, Sambasivam G, Sukumaran S. Effect of codon-optimized *E. coli* signal peptides on recombinant *Bacillus stearothermophilus* maltogenic amylase periplasmic localization, yield and activity. *J Ind Microbiol Biotechnol*. 2014;41(9):1435–42.
- Xiao L, Zhang RH, Peng Y, Zhang YZ. Highly efficient gene expression of a fibrinolytic enzyme (subtilisin DFE) in *Bacillus subtilis* mediated by the promoter of alpha-amylase gene from *Bacillus amyloliquefaciens*. *Biotechnol Lett*. 2004;26(17):1365–9.
- Ye RQ, Kim JH, Kim BG, Szarka S, Sihota E, Wong SL. High-level secretory production of intact, biologically active staphylokinase from *Bacillus subtilis*. *Biotechnol Bioeng*. 1999;62(1):87–96.

11. Nielsen H, Engelbrecht J, Brunak S, VonHeijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 1997;10(1):1–6.
12. Wu SC, Yeung JC, Duan YJ, Ye RQ, Szarka SJ, Habibi HR, Wong SL. Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. *Appl Environ Microb.* 2002;68(7):3261–9.
13. Zeigler DR, Pragai Z, Rodriguez S, Chevreux B, Muffler A, Albert T, Bai R, Wyss M, Perkins JB. The origins of 168, W23, and other *Bacillus subtilis* legacy strains. *J Bacteriol.* 2008;190(21):6983–95.
14. Kabisch J, Thuermer A, Huebel T, Popper L, Daniel R, Schweder T. Characterization and optimization of *Bacillus subtilis* ATCC 6051 as an expression host. *J Biotechnol.* 2013;163(2):97–104.
15. Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, Bertero MG, Bessieres P, Bolotin A, Borchert S, et al. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature.* 1997;390(6657):249–56.
16. Nicolas P, Maeder U, Dervyn E, Rochat T, Leduc A, Pigeonneau N, Bیدنکو E, Marchadier E, Hoebeke M, Aymersch S, et al. Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science.* 2012;335(6072):1103–6.
17. Liao Y, Huang L, Wang B, Zhou F, Pan L. The global transcriptional landscape of *Bacillus amyloquelicifaciens* XH7 and high-throughput screening of strong promoters based on RNA-seq data. *Gene.* 2015;571(2):252–62.
18. Liu X, Yang H, Zheng J, Ye Y, Pan L. Identification of strong promoters based on the transcriptome of *Bacillus licheniformis*. *Biotechnol Lett.* 2017;39(6):873–81.
19. Guan C, Cui W, Cheng J, Liu R, Liu Z, Zhou L, Zhou Z. Construction of a highly active secretory expression system via an engineered dual promoter and a highly efficient signal peptide in *Bacillus subtilis*. *New Biotechnol.* 2016;33(3):372–9.
20. Zhang K, Su L, Duan X, Liu L, Wu J. High-level extracellular protein production in *Bacillus subtilis* using an optimized dual-promoter expression system. *Microb Cell Fact.* 2017;16:32.
21. Zhou C, Xue Y, Ma Y. Characterization and high-efficiency secreted expression in *Bacillus subtilis* of a thermo-alkaline beta-mannanase from an alkaliphilic *Bacillus clausii* strain S10. *Microb Cell Fact.* 2018;17(1):124.
22. Feng Y, Liu S, Jiao Y, Gao H, Wang M, Du G, Chen J. Enhanced extracellular production of L-asparaginase from *Bacillus subtilis* 168 by *B. subtilis* WB600 through a combined strategy. *Appl Microbiol Biot.* 2017;101(4):1509–20.
23. Niu C, Liu C, Li Y, Zheng F, Wang J, Li Q. Production of a thermostable 1,3-1,4-beta-glucanase mutant in *Bacillus subtilis* WB600 at a high fermentation capacity and its potential application in the brewing industry. *Int J Biol Macromol.* 2018;107(Pt A):28–34.
24. Ma Y, Yang H, Chen X, Sun B, Du G, Zhou Z, Song J, Fan Y, Shen W. Significantly improving the yield of recombinant proteins in *Bacillus subtilis* by a novel powerful mutagenesis tool (ARTP): alkaline alpha-amylase as a case study. *Protein Expr Purif.* 2015;114:82–8.
25. Deng Y, Nie Y, Zhang Y, Wang Y, Xu Y. Improved inducible expression of *Bacillus naganensis* pullulanase from recombinant *Bacillus subtilis* by enhancer regulation. *Protein Expr Purif.* 2018;148:9–15.
26. Zhang X, Rao Z, Li J, Zhou J, Yang T, Xu M, Bao T, Zhao X. Improving the acidic stability of *Staphylococcus aureus* alpha-acetolactate decarboxylase in *Bacillus subtilis* by changing basic residues to acidic residues. *Amino Acids.* 2015;47(4):707–17.
27. Xu S, Guo Y, Du G, Zhou J, Chen J. Self-cloning significantly enhances the production of catalase in *Bacillus subtilis* WSHDZ-01. *Appl Biochem Biotechnol.* 2014;173(8):2152–62.
28. Cui W, Suo F, Cheng J, Han L, Hao W, Guo J, Zhou Z. Stepwise modifications of genetic parts reinforce the secretory production of nattokinase in *Bacillus subtilis*. *Microb Biotechnol.* 2018;11(5):930–42.
29. Zhang M, Shi M, Zhou Z, Yang S, Yuan Z, Ye Q. Production of *Alcaligenes faecalis* penicillin G acylase in *Bacillus subtilis* WB600 (pMAS) fed with partially hydrolyzed starch. *Enzyme Microb Technol.* 2006;39(4):555–60.
30. Zhu FM, Ji SY, Zhang WW, Li W, Cao BY, Yang MM. Development and application of a novel signal peptide probe vector with PGA as reporter in *Bacillus subtilis* WB700: twenty-four tat pathway signal peptides from *Bacillus subtilis* were monitored. *Mol Biotechnol.* 2008;39(3):225–30.
31. Phuong ND, Jeong YS, Selvaraj T, Kim SK, Kim YH, Jung KH, Kim J, Yun HD, Wong SL, Lee JK, et al. Production of XynX, a large multimodular protein of thermo *Anaerobacterium* sp., by protease-deficient *Bacillus subtilis* strains. *Appl Biochem Biotechnol.* 2012;168(2):375–82 (**corrected**).
32. Guo Y, Gao Z, Xu J, Chang S, Wu B, He B. A family 30 glucurono-xylanase from *Bacillus subtilis* LC9: expression, characterization and its application in Chinese bread making. *Int J Biol Macromol.* 2018;117:377–84.
33. Chen J, Chen X, Dai J, Xie G, Yan L, Lu L, Chen J. Cloning, enhanced expression and characterization of an alpha-amylase gene from a wild strain in *B. subtilis* WB800. *Int J Biol Macromol.* 2015;80:200–7.
34. Hu X, Zhang P, Miao M, Zhang T, Jiang B. Development of a recombinant D-mannose isomerase and its characterizations for D-mannose synthesis. *Int J Biol Macromol.* 2016;89:328–35.
35. Song W, Nie Y, Mu XQ, Xu Y. Enhancement of extracellular expression of *Bacillus naganensis* pullulanase from recombinant *Bacillus subtilis*: effects of promoter and host. *Protein Expr Purif.* 2016;124:23–31.
36. Wang X, Nie Y, Mu X, Xu Y, Xiao R. Disorder prediction-based construct optimization improves activity and catalytic efficiency of *Bacillus naganensis* pullulanase. *Sci Rep.* 2016;6:24574.
37. Zhang Y, Liu Y, Li Y, Liu X, Lu F. Extracellular expression of pullulanase from *Bacillus naganensis* in *Escherichia coli*. *Ann Microbiol.* 2013;63(1):289–94.
38. Messaoud EB, Ben Ammar Y, Mellouli L, Bejar S. Thermostable pullulanase type I from new isolated *Bacillus thermoleovorans* US105: cloning, sequencing and expression of the gene in *E. coli*. *Enzyme Microb Technol.* 2002;31(6):827–32.
39. Li Y, Zhang L, Niu D, Wang Z, Shi G. Cloning, expression, characterization, and biocatalytic investigation of a novel Bacilli thermostable type I pullulanase from *Bacillus* sp. CICIM 263. *J Agr Food Chem.* 2012;60(44):11164–72.
40. Chen A, Li Y, Liu X, Long Q, Yang Y, Bai Z. Soluble expression of pullulanase from *Bacillus acidopullulyticus* in *Escherichia coli* by tightly controlling basal expression. *J Ind Microbiol Biotechnol.* 2014;41(12):1803–10.
41. Clarke S, Mandelstam J. Regulation of stage II of sporulation in *Bacillus subtilis*. *J Gen Microbiol.* 1987;133(9):2371–80.
42. Coutte F, Leclere V, Bechet M, Guez J, Lecouturier D, Chollet-Imbert M, Dhulster P, Jacques P. Effect of pps disruption and constitutive expression of srfA on surfactin productivity, spreading and antagonistic properties of *Bacillus subtilis* 168 derivatives. *J Appl Microbiol.* 2010;109(2):480–91.
43. Kawabata Y, Kimura K, Funane K. Extracellular production of cyclodextrin by a protease-deficient *Bacillus subtilis* host-vector system. *Appl Microbiol Biotechnol.* 2012;93(5):1877–84.
44. Wu XC, Lee W, Tran L, Wong SL. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J Bacteriol.* 1991;173(16):4952–8.
45. Feng J, Gu Y, Quan Y, Zhang W, Cao M, Gao W, Song C, Yang C, Wang S. Recruiting a new strategy to improve levan production in *Bacillus amyloquelicifaciens*. *Sci Rep.* 2015;5:13814.
46. Thao TN, Thi DQ, Hoang TL. Cloning and enhancing production of a detergent- and organic-solvent-resistant nattokinase from *Bacillus subtilis* VTCC-DVN-12-01 by using an eight-protease-gene-deficient *Bacillus subtilis* WB800. *Microb Cell Fact.* 2013;12:79.
47. Pereira FC, Saujet L, Tome AR, Serrano M, Monot M, Couture-Tosi E, Martin-Verstraete I, Dupuy B, Henriques AO. The spore differentiation pathway in the enteric pathogen *Clostridium difficile*. *PLoS Genet.* 2013;9:e100378210.
48. Gutierrez J, Smith R, Pogliano K. SpoIID-mediated peptidoglycan degradation is required throughout engulfment during *Bacillus subtilis* sporulation. *J Bacteriol.* 2010;192(12):3174–86.
49. Willenbacher J, Rau J, Rogalla J, Syldatk C, Hausmann R. Foam-free production of surfactin via anaerobic fermentation of *Bacillus subtilis* DSM 10(T). *AMB Express.* 2015;5:21.
50. Vater J, Wilde C, Kell H. In situ detection of the intermediates in the biosynthesis of surfactin, a lipopeptide from *Bacillus subtilis* OKB 105, by whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in combination with mutant analysis. *Rapid Commun Mass Spectrom.* 2009;23(10):1493–8.
51. Zhang K, Duan X, Wu J. Multigene disruption in undomesticated *Bacillus subtilis* ATCC 6051a using the CRISPR/Cas9 system. *Sci Rep.* 2016;6:27943.
52. Zou C, Duan X, Wu J. Efficient extracellular expression of *Bacillus deramificans* pullulanase in *Brevibacillus choshinensis*. *J Ind Microbiol Biotechnol.* 2016;43(4):495–504.

53. Klosowski G, Mikulski D, Czuprynski B, Kotarska K. Characterisation of fermentation of high-gravity maize mashes with the application of pullulanase, proteolytic enzymes and enzymes degrading non-starch polysaccharides. *J Biosci Bioeng*. 2010;109(5):466–71.
54. Wei W, Ma J, Chen S, Cai X, Wei D. A novel cold-adapted type I pullulanase of *Paenibacillus polymyxa* Nws-pp2: in vivo functional expression and biochemical characterization of glucans hydrolyzates analysis. *BMC Biotechnol*. 2015;15:96.
55. Wang Y, Liu Y, Wang Z, Lu F. Influence of promoter and signal peptide on the expression of pullulanase in *Bacillus subtilis*. *Biotechnol Lett*. 2014;36(9):1783–9.
56. Shatalin KY, Neyfakh AA. Efficient gene inactivation in *Bacillus anthracis*. *FEMS Microbiol Lett*. 2005;245(2):315–9.
57. Zakataeva NP, Nikitina OV, Gronskiy SV, Romanenkov DV, Livshits VA. A simple method to introduce marker-free genetic modifications into the chromosome of naturally nontransformable *Bacillus amyloliquefaciens* strains. *Appl Microbiol Biotechnol*. 2010;85(4):1201–9.
58. Sleight SC, Sauro HM. BioBrick assembly using the in-fusion PCR cloning kit. *Methods Mol Biol* (Clifton, NJ). 2013;1073:19–30.
59. Kang J, Park K, Choi K, Park C, Kim G, Kim D, Cha J. Molecular cloning and biochemical characterization of a heat-stable type I pullulanase from *Thermotoga neapolitana*. *Enzyme Microb Technol*. 2011;48(3):260–6.
60. Wei X, Zhou Y, Chen J, Cai D, Wang D, Qi G, Chen S. Efficient expression of nattokinase in *Bacillus licheniformis*: host strain construction and signal peptide optimization. *J Ind Microbiol Biotechnol*. 2015;42(2):287–95.
61. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227(5259):680–5.
62. Radha S, Gunasekaran P. Sustained expression of keratinase gene under PxyIA and PamyL promoters in the recombinant *Bacillus megaterium* MS941. *Bioresour Technol*. 2008;99(13):5528–37.

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