Construction and development of an auto-regulatory gene expression system in *Bacillus subtilis*

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**Abstract**

**Background:** *Bacillus subtilis* is an all-important Gram-positive bacterium of valuable biotechnological utility that has been widely used to over-produce industrially and pharmaceutically relevant proteins. There are a variety of expression systems in terms of types of transcriptional patterns, among which the auto-inducible and growth-phase-dependent promoters are gaining increasing favor due to their inducer-independent feature, allowing for the potential to industrially scale-up. To expand the applicability of the auto-inducible expression system, a novel auto-regulatory expression system coupled with cell density was constructed and developed in *B. subtilis* using the quorum-sensing related promoter *srfA* (*P*<sub>srfA</sub>).

**Results:** The promoter of the *srf* operon was used to construct an expression plasmid with the green fluorescent protein (GFP) downstream of *P*<sub>srfA</sub>. The expression displayed a cell-density-dependent pattern in that GFP had a fairly low expression level at the early exponential stage and was highly expressed at the late exponential as well as the stationary stages. Moreover, the recombinant system had a similar expression pattern in wild-type *B. subtilis* 168, WB600, and WB800, as well as in *B. subtilis* 168 derivative strain 1681, with the complete deletion of *P*<sub>srfA</sub>, indicating the excellent compatibility of this system. Noticeably, the expression strength of *P*<sub>srfA</sub> was enhanced by optimizing the −10 and −35 core sequence by substituting both sequences with consensus sequences. Importantly, the expression pattern was successfully developed in an auto-regulatory cell-density coupling system by the simple addition of glucose in which GFP could not be strongly expressed until glucose was depleted, resulting in a greater amount of the GFP product and increased cell density. The expression system was eventually tested by the successful over-production of aminopeptidase to a desired level.

**Conclusion:** The auto-regulatory cell density coupling system that is mediated by *P*<sub>srfA</sub> is a novel expression system that has an expression pattern that is split between cell-growth and over-expression, leading to an increase in cell density and elevating the overall expression levels of heterologously expressed proteins. The broad applicability of this system and inducer-free expression property in *B. subtilis* facilitate the industrial scale-up and medical applications for the over-production of a variety of desired proteins.

**Keywords:** Bacillus subtilis, Protein expression system, Promoter, Quorum sensing, Auto-inducible, Auto-regulatory, Over-production, *srf* operon

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Background

*Bacillus subtilis*, a rod-shaped Gram-positive soil bacterium, has been regarded to be a “generally recognized as safe” (GRAS) microbe that can naturally secrete numerous extracellular proteins. These properties make this bacterium to be one of the most popular hosts for naturally producing a variety of enzymes and over-expressing a large number of pharmaceutical and industrial recombinant proteins of interest, such as amylase [1–4], lipase [5], alkaline PGL [6], and so on. The major advantages of *B. subtilis* compared to other protein expression hosts are that the host has high-cell-density growth and can secrete proteins directly into the cultural medium, which greatly simplifies the following steps for purification and preparation.

The most commonly used expression systems in *B. subtilis* are inducible expression systems that contain inducer-specific promoters, such as the T7 promoter; *grac* promoter; *spac* promoter induced by isopropyl-β-d-thiogalactopyranoside (IPTG); *xyIA* promoter driven by xylose; *SacB* promoter induced by sucrose; and α-amylase promoter transcribed in response to starch [4, 7, 8]. Although a variety of homologous and heterologous proteins have been successfully over-produced in *B. subtilis* under the control of these inducible promoters, a noticeable basal expression level was observed from these expression systems. Moreover, the high cost of inducers that need to be added to the medium during fermentation at a large scale would limit the industrial utilization of these protein expression systems. Recently, two strictly inducible systems, the subtilin-regulated expression (SURE) system and the *liaI*-regulated expression system (LIKE), which rely on the regulation of the promoter for the spa-box and the cell envelope stress-responsive *liaI* promoter, respectively, have been developed for scientific and industrial applications. Both of these protein expression systems are strictly controlled by the addition of lantibiotic subtilin and bacitracin, respectively, after the exponential phase of cell growth, preventing the leakage of transcription under non-inducing conditions [9, 10].

To avoid the addition of any inducers, promoters under the control of a variety of environmental stresses, such as heat shock, salt, acid, and ethanol stresses, as well as oxygen or glucose starvation [11–13], have also been used to construct systems for the expression of a variety of proteins in *B. subtilis*, none of which, could be used to over-produce industrially and pharmaceutically utilized proteins and enzymes because of the low and non-persistent expression levels of these systems as well as the difficulty of controlling protein expression. Very recently, an artificial auto-inducible system using a growth-phase promoter (*cry3Aa* promoter, transcribed during the stationary phase) and another expression system using the nutrient starvation response promoter (*pst* promoter, responded to phosphate starvation) [14, 15] were developed and characterized. Importantly, a self-inducible system for the reliable and low-cost over-production of recombinant proteins has been developed by using the manP-encoding phosphotransferase system (PTS), which is under the control of the relatively strong and strict promoter manP; manP is transcribed during a glucose-limited process in the engineered *B. subtilis* strain TQ356 [16]. The self-controlled system is suitable for high-cell-density fermentation because cell growth and over-expression are discrete at different stages.

Bacterial quorum sensing (QS) systems are cell-density-dependent regulatory networks that coordinate bacterial behavioral changes from single cellular organisms at low cell densities to multicellular organisms when their population density reaches a threshold level. The two peptides, ComX and CSF, mediate the quorum sensing control of competence and sporulation in *B. subtilis*. The accumulation of a high concentration of peptides in the late-growth phase triggers a series of gene transcription events downstream of the ComA-ComP two-component system in the cell [17], among which the *srf* operon is stimulated via a complex-regulation phosphorylated cascade (Fig. 1) [18]. As a monitor of cell density, the ComX pheromone is expressed, transported and accumulated in the broth along with cell growth. When the cells reach a certain density, the ComX pheromone reaches the obligatory concentration to activate the signal transduction system, which is composed of the two-component regulatory proteins ComP and ComA, and finally, phosphorylated ComA binds to the promoter (P_{srfA}) of the *srf* operon to activate the transcription of corresponding gene expression [19–21], leading to temporally controllable transcription.

Inspired by the transcriptional pattern of P_{srfA}, a novel gene expression system in *B. subtilis* was developed and characterized by harnessing the transcriptional properties of P_{srfA} as controlled through the response to cell density and glucose concentration during culturing. Gene expression under the control of the promoter was significantly inhibited via the addition of an appropriate concentration of glucose at the cell-growth phase until glucose was depleted, permitting the coupling of gene expression to cell density and the concentration of glucose. The functionality was examined by the efficient and controllable expression of GFP and aminopeptidase (AP). This auto-regulatory expression system could be used as an efficient tool for gene over-expression in *B. subtilis*. 

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**Note:** The text appears to have been cut off or truncated, and there is a missing reference [9, 10] that is not visible in the image. The content seems to be related to gene expression systems in *B. subtilis* and bacterial quorum sensing. The discussion includes various expression systems, including inducible and self-inducible systems, as well as regulatory mechanisms like quorum sensing. The text also mentions the development of a novel gene expression system that is controlled by cell density and glucose concentration.
Results and discussion

Expression pattern of GFP under the control of PsrfA in B. subtilis 168

In recent years, several types of expression systems that are free of inducers via a variety of transcriptional patterns have been developed in B. subtilis or other Gram-positive hosts to over-express industrially and pharmaceutically used proteins [13, 15, 16]. To expand the application of self-inducible or auto-inducible gene expression systems in B. subtilis, a novel recombinant plasmid, pBSG03, harboring a cell-density-responsive promoter, PsrfA, upstream of the reporter protein GFP was constructed, permitting the monitoring of gene expression in the hosts. To characterize the expression pattern and strength for PsrfA, pBSG03 was transformed into B. subtilis 168 and then cultivated in Terrific Broth (TB) medium for 40 h, during which the culture was sampled periodically to examine the expression level of GFP. The results demonstrate that the expression level of GFP was considerably low from the lag phase to the early exponential phase. Subsequently, the expression level increased sharply when cell growth entered the late exponential phase. Thereafter, the expression level increased gradually throughout the stationary phase until the relative fluorescence unit reached the vertex (Fig. 2a). In parallel, the intracellular proteins that were sampled in a time course were determined by SDS-PAGE analysis. The data indicated that the expression of GFP quantitatively was accumulated 9 h after fermentation, before which there were few GFP proteins that were scarcely detected on the PAGE (Fig. 2b). These results indicate that the transcription of PsrfA occurs in the late growth phase, whereas the activity of the promoter is inhibited in the early stage of cell-growth, permitting cell-density-dependent expression. The cell-density-dependent transcription of PsrfA is subjected to simple regulated mechanism of the ComA-ComP phosphorylation cascade compared to that of the recently reported gene expression system, by which the PmanP is involved in a rather complex regulatory network, making it necessary to delete the manP gene in the operon to achieve self-inducible
expression properties. Otherwise, the gene expression under \( P_{\text{manP}} \) is typically mannose-inducible in wild-type \( B.\ subtilis \) [16].

Meanwhile, the effect of four different culture media (TB medium, MM medium, IMM medium and LB medium) on \( P_{\text{srfA}} \)-mediated GFP expression in \( B.\ subtilis \) 168 (BSG101) was estimated in the following experiments. The data of the fluorescence intensity showed that the recombinant strain grown in LB medium achieved the maximal expression level, in which the expression level of GFP was approximately twofold higher than that in TB medium (Fig. 2c).

\( P_{\text{HpaII}} \) is a strong constitutive promoter, has comparable expression strength to that of \( P_{\text{43}} \), and has been used to construct an array of vectors for the over-production of recombinant proteins [22, 23]. To contrast the transcriptional level of \( P_{\text{srfA}} \) with that of \( P_{\text{HpaII}} \), plasmid pBSG04 harboring the \( P_{\text{HpaII}} \)-GFP expression cassette was constructed and transformed into \( B.\ subtilis \) 168, generating BSG102. Over the 40 h of fermentation in LB medium, each culture of the two recombinant strains was sampled periodically to measure the expression level. The data showed that the overall expression of GFP in BSG101 was higher than that of BSG102 by 1.5-fold (Fig. 2d), even though the final biomasses of the two strains were nearly the same. These results indicate that the \( P_{\text{srfA}} \) is a potentially strong promoter for gene expression in \( B.\ subtilis \).
Expression pattern of GFP in markerless deletion mutant and the protease-deficient mutants

Based on the organization of the srf operon, the native $P_{srfA}$ would also be activated, resulting in the expression of genes in the operon. To ascertain whether the background activation of the srf operon occurred simultaneously with heterologous gene expression, the markerless deletion mutant BSG168 was constructed by completely deleting the full-length of $P_{srfA}$ on the chromosome.

To compare the transcriptional features and expression levels of $P_{srfA}$ in BSG1681 with the other three types of hosts (wild-type 168, $B. subtilis$ WB600 and WB800), plasmid pBSG03 was introduced into the above hosts, generating BSG103, BSG101, BSG104, and BSG105, respectively. The levels of fluorescence intensity were monitored in the four recombinant strains throughout culturing. As expected, the transcriptional feature of $P_{srfA}$ in BSG103 was similar to that in BSG101, BSG104 and BSG105, giving a transcription pattern of cell-density-dependent auto-regulation. Noticeably, the final fluorescence intensity in BSG101, BSG103 and BSG105 was similar, but slightly higher than that in BSG104 (Fig. 3a–d). These results suggest that $P_{srfA}$ has a broad transcriptional compatibility with the cell-density-dependent pattern in various hosts. The final expression levels of GFP in the four different hosts were analyzed by SDS-PAGE, and the data were consistent with those of the fluorescence intensity (Fig. 3e).

Enhancement of the expression level by engineering the consensus sequence within the srfA promoter

Although the cell-density-dependent expression system was characterized as highly transcribed and broadly compatible in various $B. subtilis$ hosts, the duration of exponential expression under the control of the wild-type promoter was short, scarcely 10 h (Fig. 3a–d). The exponential expression phase in the other constructed auto-regulation systems starts at approximately the mid-exponential growth phase and terminated during the beginning of the stationary phase [24]. In addition, the activity of the wild-type promoter (e.g., cry3Aa), which behaves in a cell-density-dependent manner, usually disallows the over-production of a large amount of heterologous proteins to the desired level. Thus, engineering the promoters for higher capacity is the major demand for augmenting the over-production of heterologous proteins [9].

As one of the most efficient and prevalent strategies, promoter engineering has been widely used to tune the gene expression level, both for high levels of protein expression and tunable gene expression in synthetic biology [25, 26]. The major essential elements in prokaryotic promoters that mainly determine their overall transcriptional activity are the $-10$ and $-35$ consensus motifs, while other factors, such as the gene context, could also influence transcriptional activity [27]. This feature indicates that the two motifs are potential targets to be engineered within the promoter sequence. In this study, to enhance the activity of $P_{srfA}$, the sequence of the $-10$ and $-35$ motifs in $P_{srfA}$ was compared with the $\sigma^A$-dependent consensuses in $B. subtilis$. The alignment showed that the two motifs in $P_{srfA}$ were in poor homology to the consensus. Therefore, the consensus $-10$ (TATAAT) and $-35$ (TTGACT) motifs of $\sigma^A$-dependent promoters substituted for the $-10$ (TAAAAT) and $-35$ (GTGATA) motifs of the $P_{srfA}$ in plasmid pBSG03, resulting in plasmid pBSG05. After transforming pBSG05 into $B. subtilis$ 168 (generating BSG106), the cell growth and fluorescence intensity were monitored throughout culturing. After 20 h of culture, the fluorescence intensity peaked, and thereafter, the maximal level did not decrease until 80 h of culture. The maximal expression level under the control of engineered $P_{srfA}$ was approximately twofold stronger than that of the wild-type promoter (Fig. 4), demonstrating that engineering the poorly conserved $-10$ and $-35$ motifs within the promoters lead to fold increases of the transcriptional level. Despite engineering the non-inducible promoters, a set of other promoters, including inducible promoters, has been engineered to achieve a stronger over-production level through this strategy [15, 28].

Development of the cell-density-dependent expression system to an auto-regulatory system

The aforementioned expression system displayed a cell-density-dependent expression pattern. However, the cell density did not achieve a higher level than expected. According to the data referring to the expression pattern, we proposed that the initiation of a high expression level overlapped the rapid growth stage, leading to competition for energy and chemicals. Therefore, to achieve a high cell density and the desired over-production, expression initiation should be retarded until the cell density reaches a high level, avoiding mutual intervention between the two biological events. Nutrients that delayed expression while promoting cell growth were regarded as prospective candidates. A previous study revealed that the addition of glucose to the growth medium inhibits the expression of many genes, including srf operon [29].

Thus, various concentrations of glucose were chosen to appraise the influence of glucose on the expression pattern, cell biomass and expression levels. First, the cell growth, fluorescence intensity and glucose consumption rate were measured with the addition of 1, 1.5, 2, and 2.5 % concentrations of glucose. The addition of all of the concentration of glucose, except for 2.5 %, elevated
Fig. 3 Over-expression of GFP using the plasmid pBSG03 in *B. subtilis* 168 (a), BSG1681 (b), *B. subtilis* WB600 (c), and *B. subtilis* WB800 (d) was compared by determining the cell density and relative fluorescence intensity (Au/OD$_{600}$). The final expression level of GFP in the different four recombinant *B. subtilis* strains was demonstrated by SDS-PAGE analysis (e). The expression of GFP in BSG101, BSG103, and BSG105 was approximately equal, but slightly higher than that in BSG104.
of the wild-type promoter $P_{\text{srfA}}$ without adding 1.5% glucose (Fig. 5e). Moreover, the total amount of GFP fluorescence intensity and accumulative GFP protein were obtained under the control of mut$P_{\text{srfA}}$ with the addition of 1.5% glucose (Fig. 5d). The maximal expression level under the control of mut$P_{\text{srfA}}$ was approximately fourfold higher than that of the wild-type promoter $P_{\text{srfA}}$ without adding of 1.5% glucose (Fig. 2d). The highest expression level of GFP was acquired by the mut$P_{\text{srfA}}$-mediated system could be contrived to be an auto-regulatory device by the regulatory effect of glucose repression. This simple method eventually achieves a high cell density without a complex gene deletion or modification of the genome in the host. Regarding other developed auto-inducible expression systems, certain genes that are involved in the regulatory network should be disrupted or deleted to make an auto-inducible expression system while preventing native gene expression from interfering with the heterologous transcription that is mediated by the used promoters. Generally, B. subtilis under the control of $P_{\text{manP}}$, which is intrinsically a mannose-induced system, has been successfully fabricated to an auto-inducible expression by the deletion of the man$P$ gene in the PTS pathway [16].

Over-production of AP using the auto-regulatory gene expression system

To estimate the suitability of the system for the over-production of the heterologous protein, AP from B. subtilis ZJ016, plasmid pBSG06 was constructed under the control of promoter $\text{mut}P_{\text{srfA}}$. A previous report demonstrated the expression level of AP in B. subtilis WB600 under the control of $P_{\text{HpaII}}$. However, the expression and secretion levels were incomparable to those of other proteins [22, 30, 31]. Plasmid pBSG06 was introduced into B. subtilis 168, generating recombinant strain BSG107. The culture procedure was similar to that for the auto-regulatory expression mentioned above. The cell growth, AP activity, and protein over-production were measured throughout cultivation. As expected, AP expression displayed the auto-regulatory pattern coupled with a cell-density-dependent manner. The highest activity of AP that was obtained was 87.89 U/mL, and the cell-density at OD$_{600}$ achieved a level of approximately 7.87 (Fig. 6a). The expression property also be verified at the protein level by SDS-PAGE analysis (Fig. 6b). Those results indicate that $\text{mut}P_{\text{srfA}}$-mediated system could be used to express heterologous proteins in B. subtilis.

Conclusions

In this study, a novel auto-regulatory gene expression system coupled to cell density in B. subtilis was constructed and developed using the srfA promoter that is associated with triggering quorum sensing. Using the expression system, expression was separated by the cell growth phase and high expression phase, during which the addition of glucose not only augmented the two-phase expression profile and enabled auto-regulation but also increased the final cell density. In addition, the expression strength was remarkably enhanced by engineering the −10 and −35 region via substitution to the highly evolved regions.
Fig. 5  The effect of different concentrations of glucose on the P_{\sigmaA} and m\textnuP_{\sigmaA} controlled expression of GFP. a The cell density profiles for BSG101 harboring wild-type PsrfA in LB medium with the addition of 1 % (solid rectangle), 1.5 % (solid cycle), 2 % (solid triangle), and 2.5 % (solid inverse triangle) glucose. Medium without the addition of glucose was used as the control group (solid pentangle). b Glucose consumption profiles at 8, 12, 16, and 20 h with different initial concentrations. c The fluorescence intensity profiles that were detected in BSG101 with different concentrations of glucose corresponded to those in a. d The fluorescence intensity and the cell density that were detected in recombinant strain BSG106 that harbored m\textnuP_{\sigmaA}, in which the intrinsic sequences of $-10$ and $-35$ were replaced by the corresponding conserved sequences for $\sigmaA$-dependent promoters. e Comparison of the highest fluorescence intensity and the corresponding GFP expression level with or without the addition of glucose in B. subtilis 168 under the control of wild-type P_{\sigmaA} and m\textnuP_{\sigmaA}. Sample 1 represents the BSG101 without the addition of glucose, which was designated as the control. Samples 2–5 represent strain BSG101 with the addition of 1, 1.5, 2, and 2.5 % glucose, respectively. Sample 6 represents strain BSG106 (harboring m\textnuP_{\sigmaA}) with the addition of 1.5 % glucose. All of the experiments were independently performed in triplicate.
conserved sequences. Furthermore, aminopeptidase was successfully over-produced as the test protein using the newly constructed gene expression system, achieving a 1.7-fold over-expression level compared to that under the control of the generally regarded strong promoter $P_{\text{rtpall}}$ [30]. These features of the auto-regulatory gene expression system give the system great potential for the over-production of enormous high-value-added proteins at the industrial scale.

Methods

Strains, plasmids and growth conditions

The bacterial strains and plasmids that were used in this study are listed in Table 1. *E. coli* JM109 was used as a host for gene cloning. *B. subtilis* 168 and engineered *B. subtilis* BSG1681 were used for the gene expression and gene integration experiments. The media used in this study were glucose minimal medium (MM) [32], improved minimal medium (IMM, adding 0.02 % Casamino acids to MM medium), Luria–Bertani medium (LB), and TB. All of the strains were cultured in the appropriate medium with aeration at 37 °C. When appropriate, *B. subtilis* growth media was supplemented with kanamycin (5 μg/mL) and *E. coli* growth media with ampicillin (10 μg/mL). The cell density was determined by measuring the OD$_{600}$ with a UV-1800/PC spectrophotometer (Shanghai, MAPADA Instrument Co., Ltd., China).

Recombinant DNA techniques

Plasmid construction was performed in *E. coli*, and extraction was processed following the standard procedure as previously described [33]. Recombinant plasmids were transformed into *B. subtilis* as previously described [30]. Enzymes were obtained from TOYOBO (Osaka, Japan), TaKaRa (Dalian, China), or New England Biolabs (Beijing, China) and were used according to the manufacturers’ protocols. The primers used in this study are listed in Table 2. PCR was performed using KOD DNA polymerase (Osaka, Japan). All of the recombinant plasmids that were constructed in this work were confirmed by DNA sequencing (Shanghai Sangon Biotech Co., Ltd., China).

Construction of plasmids and mutant strains with markerless gene deletion

Plasmid construction was carried out using the Sequence and Ligation Independent Cloning (SLIC) method, with few modifications [34]. The primers that were used to amplify the donor sequence were designed to have 30-bp flanking regions that are homologous to the receptor plasmid insertion regions, and the receptor plasmid was linearized by inverse PCR. The main procedures used for construction are shown in Fig. 7. The plasmids constructed in this study were *E. coli*–*B. subtilis* shuttle vectors.

To construct pBSG01, Fragment 1, which is 2.2 kb and comprises the ampicillin resistance gene (*bla*) and replication region (pBR322 ori) and was flanked by the corresponding homology to Fragment 2, was amplified from pUC19 with primers P1 and P2. Concomitantly, Fragment 2 carrying the replication protein (Rep), kanamycin resistance gene (*neo*), and bleomycin resistance gene (*ble*), which was flanked by the corresponding homology of Fragment 1, was amplified from plasmid pMA09 using primers P3 and P4. These two fragments were mixed at the molar ratio of 1:2 prior to treatment with T4 DNA polymerase. After a 2.5-min treatment, the mixture was
incubated on ice for 10 min, allowing for the two fragments to be annealed, after which the mixture was transformed to *E. coli* JM109 competent cells [35]. The resulting plasmid was termed pBSG01.

To introduce promoter PsrfA into the plasmid pBSG01, the 0.6-kb PsrfA flanked by a 30-bp homology sequence upstream and downstream of the inserted position in pBSG01 was amplified from the chromosome of *B. subtilis* 168 using primers P5 and P6. Plasmid pBSG01 was then linearized using inverse PCR with primers P7 and P8. The two fragments were joined according to the steps described above. The resulting plasmid was termed pBSG02. To insert GFP downstream of PsrfA in pBSG02, the gfp gene was amplified from pBS1154 with primers P9 and P10, flanked by the 30-bp homology sequence corresponding to the upstream and downstream sequences at the inserted position. Meanwhile, pBSG02 was linearized by inverse PCR with primers P11 and P2. These two fragments were joined by the SLIC method mentioned above, generating plasmid pBSG03 for expression in *B. subtilis*.

To construct the comparative plasmid pBSG04 under the control of promoter HpaII (P_HpaII), P_HpaII was inserted into pBSG03 by replacing PsrfA using mega-primer PCR with a whole plasmid protocol using primers P7 and P12 [36]. Plasmid pBSG05, harboring the mutant PsrfA, was constructed with primers P13 and P14 using pBSG03 as the template through inverse PCR. Then, the endonuclease DpnI was used to cut the template plasmid. To express aminopeptidase (AP) under the control of PsrfA, plasmid pMA05 was used as the template to amplify AP with primers P15 and P16, and pBSG02 was linearized with primers P11 and P2. Plasmid pBSG06 was obtained by processing these two fragments in the same manner as for pBSG02. All of the plasmid constructs were verified by DNA sequencing.

The markerless deletion of genes on the chromosome of *B. subtilis* 168 was performed as previously described, with some modification [37, 38]. The sequences of the *B. subtilis* dif site (dif *B. subtilis*: ACTTCCTAGAATATATATATGTAAACT) were used in this study.

To delete the promoter region in the srf operon from the *B. subtilis* 168 chromosome, two tripartite fragments

### Table 1 Strains and plasmids used in this study

| Strains and plasmids | Relevant properties | References |
|----------------------|--------------------|------------|
| **Strains**          |                    |            |
| *E. coli* JM109      | recA1, endA1, gyrA96, thi-1, hsdR17(rK- mK+), e14^−(mcrA^+), supE44, relA1, Δ(lac-proABF') [traD36, proAB^+], lacD, lacZΔM15] |            |
| *B. subtilis* 168    | trpC2              |            |
| WB600 (168)ΔnprEΔaprΔlepΔbrpΔnprΔlepβ | [40] |
| WB800 (168)ΔnprEΔaprΔlepΔbrpΔnprΔlepβ | [41] |
| BSG1681 (168)ΔPsrfA | This study         |            |
| BSG101 168, pBSG03 (PsrfA-gfp) | This study |
| BSG102 168, pBSG04 (PsrfA-gfp) | This study |
| BSG103 BSG1681, pbSG03 (PsrfA-gfp) | This study |
| BSG104 WB600, pBSG03 (PsrfA-gfp) | This study |
| BSG105 WB800, pBSG03 (PsrfA-gfp) | This study |
| BSG106 168, pBSG05 (mutPsrfA-gfp) | This study |
| BSG107 168, pBSG06 (mutPsrfA-ap) | This study |
| **Plasmids**         |                    |            |
| P7Z6                 | zeo^+ bl^− Cre/lox | [38]       |
| pUC19                | pUC origin P tet, Ap^− | Takara |
| pMA09                | E. coli-B. subtilis shuttle vector, Ap^−, Apr, PHpaII | [30] |
| pMA05                | E. coli-B. subtilis shuttle vector, Ap^−, Apr, PHpaII-AP | [30] |
| pbSG01               | bl^−, ne^−, E. coli-B. subtilis shuttle vector | This work |
| pbSG02               | pBSG01 ligated with PsrfA | This study |
| pbSG03               | pBSG02 with GFP ligated downstream of PsrfA | This study |
| pbSG04               | pBSG03 with PsrfA replaced by PsrfA | This study |
| pbSG05               | pBSG03 with mutPsrfA | This study |
| pbSG06               | pBSG02 with AP ligated downstream of mutPsrfA | This study |

*a* mutPsrfA: the −10 region (TAAACT) and −35 region (GTGATA) of PsrfA was replaced by the consensus −10 region (TATAAT) and −35 region (TTGACT) of σ^A^-dependent promoters.
(Fig. 8) were amplified from the B. subtilis genome using the primers that are listed in Table 2. The specific procedure is described as follows. The up-sequence, which consisted of the approximately 1-kb homologous fragment upstream of P_srfA, the dif B. subtilis site, and 6-bp homologous region to the 5′ end of the gene zeo, was amplified by PCR with primers P17 and P18. Similarly, the down-sequence, which consisted of a 5-bp homologous fragment to the 3′ end of the gene zeo, the dif B. subtilis, and the approximately 1-kb homologous fragment downstream of the gene P_srfA, was amplified with primers P19 and P20. Next, the zeocin resistance gene zeo was amplified from plasmid p7Z6 using primers P21 and P22, flanked by a 5-bp homology to the sequence in the chromosome followed by the 28-bp dif sequence at the 5′ terminus and the 28-bp dif sequence followed by a 5-bp homology to the chromosome. These three fragments were assembled by fusion PCR. The resulting 2.5-kb fragment was then sequenced and transformed into the B. subtilis 168 competent cells. The integrants were selected on LB agar medium containing 25 μg/mL zeocin. The positive clones were selected before inoculating the LB broth in the absence of antibiotics and were incubated for approximately 36 h to produce zeocin-sensitive recombinant clones. Then, the culture was identified by replica agar plating with and without the selective antibiotic. Finally, the clones that grew on the agar plate in the absence of antibiotics were selected in liquid medium for identification by PCR using primers P17 and P20. The positive mutant strain that was deficient in P_srfA was named BSG1681 (B. subtilis 168: ΔP_srfA).

GFP fluorescence assay
A single colony of the appropriate B. subtilis strain picked on LB agar plates was inoculated in 5 mL of LB liquid medium and was cultured overnight for more than 12 h to serve as a preculture. Then, 0.6 mL of preculture was transferred into 250-mL shaking flasks that were loaded with 30 mL of LB liquid medium to cultivate for 3 days, during which the culture medium was sampled every 2 h. After cultivation, the cells were harvested by centrifugation, washed by PSB buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.5) for three times, and resuspended in an appropriate diluted ratio. Then, a final volume of 200 μL of the cell suspension was transferred to each well, and the fluorescence value (expressed as Au) was recorded. The fluorescent activity of GFP was monitored via fluorescence spectroscopy on a black flat 96-well plate using a Synergy™ H4 multimode microplate reader (BioTek Instruments, Inc., USA). The excitation wavelength was

| Primers | Sequences |
|---------|-----------|
| P1      | CTCTTCCGCTTCCTGCCCTACGACTCGC |
| P2      | CGGATTCTTCTCTTAGGACCTGTCGCGG |
| P3      | CGGGCTTCGAGGAGGGAGGGGAGGAAAG |
| P4      | CGGGACAGATGGTAAAGGAGAAGAGAAATACCGGGCTTATTGTAGTCTCATCCATG |
| P5      | CAGGTTATGCTGGGAGGGAGGAGAGGGGAGGAAAG |
| P6      | CTGGCTATGAGGGAGGAGAAGAGGGGAGGAAAG |
| P7      | CGGATGTCTGCCACACTCAGC |
| P8      | CATTAGTACCTCTCCCTAATC |
| P9      | CTGGATTAAGGAGGAGAAGAATC |
| P10     | CTGGATTAAGGAGGAGAAGAAGAGGAGAAGAGGAAAG |
| P11     | CATTAGTTACCTCTTGACCTCACACCCCTTTGGATATC |
| P12     | ATGGAGCTTGCTGTCAGGGAGGAGAAGAGGAAAG |
| P13     | ACTGGGATGAGGGAGGAGAAGAGGAAAG |
| P14     | ACTGGGATGAGGGAGGAGGAGGAGGAAAG |
| P15     | ACTGGGATGAGGGAGGAGGAGGAGGAAAG |
| P16     | CGGAGTACCTCTTGACCTCACACCCCTTTGGATATC |
| P17     | CGGATGTCTGCCACACTCAGC |
| P18     | ATGGAGCTTGCTGTCAGGGAGGAGAAGAATC |
| P19     | ACTGGGATGAGGGAGGAGGAGGAGGAAAG |
| P20     | ATGGAGCTTGCTGTCAGGGAGGAGAAGAATC |
| P21     | TTGAATCGGTCATAGGATGAGGAGGAGGAGAAGAATC |
| P22     | TGGATGAGGGAGGAGGAGGAGGAGGAAAG |

* Homologous sequences were underlined, and dif B. subtilis sequence was shown in bold.
495/9 nm, and the emission wavelength was 525/9 nm with gain 80. The determination of the GFP expression was calculated with the fluorescence intensity divided by the OD600 that was previously measured [39].

The relative fluorescence intensities which reflected the expression levels were calculated according to the method described by Toymentseva et al. [10]. The relative fluorescence intensity (Au/OD600) of three parallel samples of the recombinant B. subtilis strains harboring empty vector pBSG01 were averaged and subtracted from that of the recombinant strains harboring plasmids with GFP at the consistency time of cultivation. Growth was monitored by measuring the absorbance at 600 nm. The data were averaged from three independent samples of the same time points.

**Heterologous expression of aminopeptidase and enzyme activity analysis**

A fresh overnight culture of the recombinant B. subtilis strain BSG107 harboring pBSG06 under the control of mutant P_{srfa} (mutP_{srfa}) was inoculated into 250-mL shaking flasks that were loaded with 30 mL of LB liquid medium, the optical density of which was adjusted to OD600 0.2. Then, the culture was cultivated at 37 °C with rigorous shaking for 2 days, and sampling was implemented throughout the fermentation process with an interval of 2 h. Thereafter, crude AP in the fermentation supernatant was obtained by removing recombinant B. subtilis cells via centrifugation (5 min, 10,000 x g). AP activity was determined via a previously described method [23]. One unit of activity was defined as the
amount of enzyme that released 1 µmol L⁻¹ p-nitroaniline min⁻¹ at 37 °C (ε₄₀₅ nm = 9.98 L mmol⁻¹ cm⁻¹). The results are the averages of triplicate assays.

Protein analysis and SDS-PAGE
After cultivation for appropriate recombinant B. subtilis, the cells were harvested by centrifugation. The pelleted cells were then re-suspended in PBS buffer prior to disruption by ultra-sonication on ice. The crude cell extracts were separated by centrifugation, the supernatant was analyzed by SDS-PAGE according to the standard procedure, and the gels were subsequently stained by Coomassie brilliant blue R250 staining. The concentration of the total proteins was determined by the Bradford assay and equal amounts of total protein were loaded onto SDS-PAGE gels.
Abbreviations

$P_{srfA}$: the promoter of the $srf$ operon; PTS: phosphotransferase system; QS: quorum sensing; AP: aminopeptidase; GFP: green fluorescent protein; SLIC: sequence and ligation independent cloning; bIa: ampicillin resistance gene; pBR222 or: replication region, Fep: replication protein, neo: kanamycin resistance gene; bIe: tetracycline resistance gene; $P_{rep}$: promoter of $p$br322 ori: replication region; Rep: replication protein; kan: kanamycin resistance gene; $P_{neo}$: promoter of neo; Sp: the signal peptide of aminopeptidase; $m_{srfA}$: the mutant of promoter $P_{srfA}$.

Authors' contributions

CG constructed the plasmids, the variant strain, accomplished the determination of the recombinant expression system, analyzed the data and wrote the manuscript. L.Z, J.C, J.G, X.H were participated in the determination of the recombinant expression system, analyzed the data and wrote the manuscript. Z.L, J.C, J.G, X.H were participated in the determination of expression pattern using GFP reporter gene. Q.X participated in the experiment of expressing aminopeptidase. Z.C.Z conceived the idea, designed this study, analyzed the data and wrote the manuscript. W.C conceived the idea, designed this study, analyzed the data and wrote the manuscript. Z.L, J.C, J.G, X.H were participated in the determination of expression pattern using GFP reporter gene. Q.X participated in the experiment of expressing aminopeptidase. Z.C.Z conceived the idea, designed this study, analyzed the data and wrote the manuscript. W.C conceived the idea, designed this study, analyzed the data and wrote the manuscript. Z.L, J.C, J.G, X.H were participated in the determination of expression pattern using GFP reporter gene. Q.X participated in the experiment of expressing aminopeptidase.

Compliance with ethical guidelines

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Competing interests

The authors declare that they have no competing interests.

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