The multifunctionality of expression systems in *Bacillus subtilis*: Emerging devices for the production of recombinant proteins

Caio Coutinho de Souza¹, Jander Matos Guimarães², Soraya dos Santos Pereira³,⁴,⁵ and Luis André Morais Mariúba¹,⁵,⁶,⁷

¹Programa de Pós-Graduação em Biotecnologia da Universidade Federal do Amazonas – UFAM, Manaus, AM 69067-005, Brazil; ²Centro Multissuário de Análise de Fenômenos Biomédicos (CMABio) da Universidade do Estado do Amazonas (UEA), Manaus, AM 69065-00, Brazil; ³Fundação Oswaldo Cruz (FIOCRUZ) Unidade de Rondônia, Porto Velho-RO 76812-245, Brazil; ⁴Programa de Pós-Graduação em Biologia Experimental, Fundação Universidade Federal de Rondônia-PGBIOEXP/UNIR, Porto Velho-RO 76801-974, Brazil; ⁵Programa de Pós-Graduação em Biologia Celular e Molecular, Instituto Oswaldo Cruz, IOC, Rio de Janeiro 21040-360, Brazil; ⁶Instituto Leónidas e Maria Deane (ILMD), Fundação Oswaldo Cruz (FIOCRUZ), Manaus, AM 69057-070, Brazil; ⁷Programa de Pós-Graduação em Imunologia Básica e Aplicada, Instituto de Ciências Biológicas, Universidade Federal do Amazonas (UFAM), Manaus, AM 69067-00, Brazil

Corresponding author: Luis André Morais Mariúba. Email: andre.mariuba@fiocruz.br

**Impact statement**

The use of *Bacillus subtilis* to produce recombinant proteins with medical, biotechnological, and industrial value is a growing activity that presents an enormous potential for exploration. This host has been standing out and gaining notability because it offers laboratory safety and excellent yields. Such elements stimulate more and more the development of expression systems that use particular and unconventional genetic engineering strategies. We revised the wide availability and screening of several efficient promoters, in addition to the use of economically viable chemical inducers and self-inducible expression systems, which are increasingly in demand today for their practicality. We hope this article clarifies recent systems that comprise the technological arsenal available for this expression platform and that may be useful to various research groups as reliable alternatives to the worldwide need for bioactive produced by a scalable source of production.

**Abstract**

*Bacillus subtilis* is a successful host for producing recombinant proteins. Its GRAS (generally recognized as safe) status and its remarkable innate ability to absorb and incorporate exogenous DNA into its genome make this organism an ideal platform for the heterologous expression of bioactive substances. The factors that corroborate its value can be attributed to the scientific knowledge obtained from decades of study regarding its biology that has fostered the development of several genetic engineering strategies, such as the use of different plasmids, engineering of constitutive or double promoters, chemical inducers, systems of self-inducing expression with or without a secretion system that uses a signal peptide, and so on. Tools that enrich the technological arsenal of this expression platform improve the efficiency and reduce the costs of production of proteins of biotechnological importance. Therefore, this review aims to highlight the major advances involving recombinant expression systems developed in *B. subtilis*, thus sustaining the generation of knowledge and its application in future research. It was verified that this bacterium is a model in constant demand and studies of the expression of recombinant proteins on a large scale are increasing in number. As such, it represents a powerful bacterial host for academic research and industrial purposes.

**Keywords:** Bacillus subtilis, promoters, recombinant protein, expression systems, induction, self-induction

**Experimental Biology and Medicine** 2021; 246: 2443–2453. DOI: 10.1177/15353702211030189

**Introduction**

*Bacillus subtilis* is a Gram-positive bacteria host qualified by the Food and Drug Administration (FDA) as being a generally safe microorganism (GRAS) that is free of exotoxins and endotoxins.¹ The reading of its codons is remarkably diverse and contributes to the expression of heterologous genes that do not depend on additional steps.² The applications of *B. subtilis* include food fermentation, cell division...
studies, development of biofilms, production of secondary metabolites, and it is also a potential host for vaccine production, and a delivery vehicle.3–7

In addition, it has established itself as a robust cell host and an efficient technological platform for the expression of bioactive and high-yield protein production, with the potential for scaling in a bioreactor. The main challenge in the production of recombinant proteins in *B. subtilis* is that of how to choose a relevant expression system.8 Different expression systems have already been developed, and proteins from prokaryotic or eukaryotic organisms have been synthesized and purified.9 The best strategy depends on the protein to be expressed, though always with the aim of cost reduction, efficiency, and high-yield production.10 New strategies include the use of constitutive promoters, double promoters, functional synthetics that are capable of directing transcription, efficient signal peptides, and inducible and self-inducing expression systems.11,12

These improved characteristics, together with the growing demand for biotechnology products, contribute to the increase in the use of this bacterium, thus endorsing its widespread use in the field of genetic and metabolic engineering.13,14 Since the first report of heterologous overproduction of recombinant proteins in this strain, the mass production of an infinity of valuable bioactive molecules has been performed on a relatively large scale.15

Recognizing the importance of metabolic engineering, systematic biology, synthetic biology, and evolution-based engineering in *B. subtilis*, this review first summarizes the importance of technical knowledge and the construction of improved strains suitable for industrial production. Next, we present an overview of the *B. subtilis* secretion system and its crucial role in heterologous protein production. Finally, we review the versatility of the application of promoters and others expression mediators as strategies that aim at production efficiency, also recent advances inducer-free expression vectors construction.

Therefore, this review covers the latest and the principal expression systems developed in *B. subtilis* and the strategies inherent to the multivalence of this expression platform and confirm its wide capacity and its recognition as an important host in the modern biotechnological industry. The bibliographic survey of indexed publications was performed using the PUBMED, Google Scholar, and “Periódico Capes” databases. Only publications in the English language and from 2015 to 2020 were included. The keywords used for the search were “Bacillus subtilis”, “Expression Systems”, “Promoters”, “Induction”, “Self-induction”, and “Recombinant proteins”.

The *B. subtilis* expression platform

*B. subtilis* is constantly referred to as a reference system and a collection of biochemical, genetic, and physiological knowledge of Gram-positive bacteria.16 Its sequencing was carried out by Kunst *et al.*17 in 1997 using strain 168 and involved more than 30 laboratories worldwide. Subsequently, other research groups carried out a complete sequencing of the genome that included criteria that ensured the characterization of its genes and products.18

Bacterial preservation centers around the world have conserved numerous strains of *B. subtilis*, including the auxotrophic strains 23, 122, 160, 166, and 168, which were derived originally from *B. subtilis* Marburg.19 The 168 strain has been extensively used in academic research and industrial production.20 Given the availability of an increasing number of cloning vectors and mutant host lines that are provided by the Bacillus Genetic Stock Center (BGSC),21 many strains can be transformed using systematic metabolic engineering to obtain mutant strains that meet production requirements.22 For example, *B. subtilis* WB600 and *B. subtilis* WB800 were constructed from *B. subtilis* 168 by knocking out six and eight protease genes, respectively.20,23 Although some proteases may be of interest to the industry, their activity generally limits the overall efficiency of heterologous protein production.23 However, the use of these strains becomes attractive, as it partially solves the problem of proteolysis of secreted target proteins.24

These sequencings and the emergence of powerful genetic tools culminated in the rise in popularity of *B. subtilis*, mainly in the industrial segment.25 It is one of the most frequently used bacterial hosts for producing high-value recombinant proteins.26 Furthermore, there has been an unprecedented increase in the number of molecules produced in this model, which has provided it with a prominent role in the industrial production of proteins worldwide and has transformed *B. subtilis* into the most studied species of the genus *Bacillus*.23 Several examples are shown in Table 1.

The production of some protein classes can be difficult, for example when it comes to proteins with multiple subunits or with a high molecular weight that faces problems relating to transport across the cell membrane. This requires the use of alternative transport routes which the determining mechanisms related to these types of non-classical secretory pathways are still limiting and unclear.65,66 The sequences of synthetic genes also need to be optimized to achieve the best possible expression,67 since, in the same way, that occurs in other species, some codons are rarely used, and the selection of synonymous codon is restricted by the efficiency of translation. Highly expressed genes are almost always more dependent on the tRNA content and tend to have a strong predisposition of codons usage.68,69 From a structural point of view, unlike other bacterial hosts, *B. subtilis* does not have an external membrane or periplasmic space, which gives it a greater capacity for protein secretion directly into its surrounding environment.20 And based on proteomic analysis studies, *B. subtilis* has already demonstrated the potential to export approximately 300 proteins.70

The secretion system in *B. subtilis*

The steps for producing recombinant proteins in *B. subtilis* are relatively simple (Figure 1(a)). To ensure that the proteins are properly directed to the pathways and thus initiate the translocation process, specific signal peptides are present at the N-terminus of each protein and must be cleaved by a peptidase for later release of the mature protein to be exported to the medium, retained in the cell wall, or
Table 1. Expression systems developed in Bacillus subtilis from 2015 to 2020.

| Promoter | Type          | Plasmid | Inducer | Host              | Recombinant proteins                                      | Concentration obtained     | References |
|----------|---------------|---------|---------|-------------------|-----------------------------------------------------------|---------------------------|------------|
| P<sub>43</sub> | Constitutive   | pHT01   | None    | B. subtilis WB800N | Trehalose synthase (TreS)                                  | 23080.6 ± 1119.4 U/L      | Liu et al.<sup>27</sup> |
| P<sub>43</sub> | Constitutive   | pWB980-mt2303 | None    | B. subtilis SC6K | Trypsin (GM2938)                                           | 1622.2±0/12 U/mL           | Wang et al.<sup>28</sup> |
| P<sub>43</sub> | Constitutive   | pUC980-2 | None    | B. subtilis WB800 | Alkaline protease (pro1)                                   | 504 U/mL                   | Zhao et al.<sup>29</sup> |
| P<sub>43</sub> | Constitutive   | pP43NK  | None    | B. subtilis WB600 | B-like asparaginase                                        | 374.9 U/mL                 | Feng et al.<sup>30</sup> |
| P<sub>43</sub> | Constitutive   | PhTA4   | None    | B. subtilis 168   | Pulullanase                                                | 24.5 ± 0.5 U/mL            | Song et al.<sup>31</sup> |
| P<sub>43</sub> | Constitutive   | pHT43   | (ATCC 33,712) | None | Microbial transglaminase                                   | 63.0 ± 0.6 mg/L            | Mu et al.<sup>32</sup> |
| P<sub>43</sub> | Constitutive   | pucb19  | None    | B. subtilis WB600 | Pullulanase organophosphorus                               | N/A                        | Yu et al.<sup>33</sup> |
| P<sub>43</sub> | Constitutive   | pWB980-mt2303 | None    | B. subtilis 1751 | Aminopeptidase                                             | 87.89 U/mL                 | Guan et al.<sup>34</sup> |
| P<sub>43</sub> | Constitutive   | pUC980-2 | None    | B. subtilis 1751P | x-Amylase                                                  | 1089 U/mL                  | Chen et al.<sup>35</sup> |
| P<sub>43</sub> | Constitutive   | PhTB110 | None    | B. subtilis 1751P | d-Psicose 3-epimerase                                      | N/A                        | He et al.<sup>36</sup> |
| P<sub>43</sub> | Constitutive   | pCGT4   | None    | B. subtilis CCTCC | A-(cyclodehydroxyacyltransferase)                         | 57.12 U/mL                 | Zhang et al.<sup>37</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis WB600 | Pullulanase                                                | 26.5 U/mL                  | Deng et al.<sup>39</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | DSM13 mannan (endo-1,4-mannosidase)                       | 2207 U/mL                  | Song et al.<sup>40</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Lipase LipA                                                | 287.8 U/mL                 | Ma et al.<sup>41</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Amyl; AmyS                                                 | 1352 and2300 U/mL          | Chen et al.<sup>42</sup> |
| P<sub>43</sub> | Constitutive   | pMA4    | None    | B. subtilis ATCC 33,712 | Human epidermal growth factor (hEGF)                 | 360 ± 9.41 mg/L            | Su et al.<sup>43</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Poly-L-glutamic acid                                       | 0.13 C-mol C-mol<sup>-1</sup> | Halsmehl et al.<sup>44</sup> |
| P<sub>43</sub>'-P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Riboflavin                                                | OD<sub>600</sub> = 0.5     | Correa et al.<sup>45</sup> |
| P<sub>43</sub> | Constitutive   | pMCU7-T5W | None    | B. subtilis WB800 | Man<sup>-t</sup>-mannosidase (M<sup>-</sup>-mannosidase)   | 17.02 U/mg                 | Kang et al.<sup>46</sup> |
| P<sub>43</sub> | Constitutive   | pUC980-2 | None    | B. subtilis 1751P | Aspartase (aspa)                                          | 13.11 U/mL                 | Han et al.<sup>47</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Aminopeptidase (AP)                                       | 205 U/mL                   | Guan et al.<sup>48</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Neopullulanase                                            | 45 U/mg                    | Hervia et al.<sup>49</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | r-glucorindase                                             | 7.5 U/mL                   | Cui et al.<sup>50</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | r-glucorindase                                             | 200 ng/mL                  | Han et al.<sup>51</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Strephi-SUMO-PhoA                                         | 10 mg                      | Heinrich et al.<sup>52</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Nanobodies                                                | 15 to20 mg                 | Yang et al.<sup>53</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | MAK33-3L                                                  | 2 mg L<sup>-1</sup>         | Schiedler et al.<sup>54</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Cycopin AD                                                | 26.4 mg/L                  | Zhang et al.<sup>55</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | PR-FO                                                    | 7 mg                       | Zhang et al.<sup>56</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Transglaminase (TG)                                       | 2.6 U/mg                   | Fu et al.<sup>57</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | 8BPMP                                                    | 3.16 g/L                   | Sun et al.<sup>58</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Pulullanase                                                | 2.74 mg mL<sup>-1</sup>    | Pang et al.<sup>59</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | GFP                                                       | 9.1 U/mL                   | Promchaid et al.<sup>60</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | L-theanine                                                | 53 g l<sup>-1</sup>         | Yang et al.<sup>61</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | AQA7                                                      | 1.27 µg                    | Ramos et al.<sup>62</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | HRV3C                                                    | 8065 U/mg                  | Le et al.<sup>63</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Creatinase                                                | 14.19 U/mL                 | Tao et al.<sup>64</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P |r-glucorindase (GusA) and nattokinase (NK)                | 4.37 ± 15.8 FU/mL          | Han et al.<sup>65</sup> |
| P<sub>43</sub> | Constitutive   | pMA0911 | None    | B. subtilis 1751P | Serine protease alkaline (BcaPRO)                        | 27.860 U/mL                | Liu et al.<sup>66</sup> |
B. subtilis contains a strict quality control system for protein-dependent translation system and performed by intracellular and extracytoplasmic chaperones, cell wall proteases, and extracellular proteases. And several different protein export systems have been identified in this bacteria so far.

The general secretion pathway (Sec)-dependent transport system and the “Twin-arginine” (Tat) translocation system are generally studied and used for the secretion of recombinant proteins in the extracellular medium in B. subtilis. The general secretion pathway (Sec) (Figure 1(b)) includes components that convert energy in the form of ATP and transmembrane protons as the driving force to direct proteins through channels incorporated in the membrane. The general secretion system (SeC) is considered the main route for the secretion of proteins in the growth medium and has the characteristic of transporting proteins across the cytoplasmic membrane in an unfolded or weakly folded conformation. In the co-translational export mode, the protein is synthesized ribosomally as a precursor protein containing a signal peptide in the N-terminal region, being helped to maintain its competent state of translocation by cytoplasmic chaperones, with the subsequent recognition of the signal peptide by the particle signal recognition (SRP), as well as targeting membrane translocases. The targeting of the pre-protein by this SRP complex also involves the presence of the FtsY protein, a highly conserved GTPase that also acts in targeting the SecYEG transmembrane channels. In this way, the PrsA lipoprotein is necessary for the subsequent folding of the mature protein, in the assistance for providing stability, and avoiding non-productive interactions with the cell wall. Another strategy in the post-translation export mode, SecA transfers the precursor proteins to the membrane.
translocation channel, then the SecA motor protein translocates the pre-proteins through SecYEG using metabolic energy from ATP hydrolysis.\textsuperscript{86}

In Tat (Figure 1(c)), before the translocation of the recombinant protein through the cytoplasmic membrane, they become completely bent in the cytoplasm with the help of intracellular chaperones, in a strategy capable of reducing proteolytic degradation.\textsuperscript{21} On the other hand, in the Tat-dependent pathway, secreted proteins usually contain a typical highly conserved cleavable signal peptide that directly affects secretion.\textsuperscript{81} The precursor protein strongly folded by intracellular chaperones is transported through a channel formed by the translocase complex.\textsuperscript{82} In \textit{B. subtilis}, two separate translocation systems work in parallel: TatAdCd and TatAyCy. TatAdCd is expressed as a translocase under phosphate-deprived conditions, while TatAyCy is expressed constitutively.\textsuperscript{20,71} TatAd and TatCd combine to form the TatAdCd complex, while TatAy and TatCy form the TatAyCy complex. As shown in Figure 1(d), proteins also can be exported to the medium via ATP binding cassette transporters (ABC).\textsuperscript{71} In contrast (Figure 1(e)), small numbers of proteins are exported by the pseudopilin (Com) export route.\textsuperscript{73}

### The constitutive promoters in \textit{B. subtilis}

Notably, many efforts have been made to identify strong promoters for transcription control. In this sense, P\textsubscript{43} is a promoter that meets widely used requirements. P\textsubscript{43} was first described in the studies of Wang and Doi in 1984 and best characterized by Song and Neuhard in 1989.\textsuperscript{53,84} It was used to produce methyl parathion hydrolase and to develop an expression system for \textit{B. subtilis} based on food-grade expression plasmids.\textsuperscript{85,86}

In a recent study, the P\textsubscript{43} promoter was successfully fused with other promoters to construct a series of plasmids to extend the screening of signal peptides (SPs) and to obtain promoters suitable for secretion of BacPro (alkaline serine protease) from \textit{B. clausii}. The signal peptide “DacB” and the dual promoter “P\textsubscript{Bam}P\textsubscript{Bam}” demonstrated the best performance, with a high expression level of BacPro (27,860 U/mL).\textsuperscript{64} Liu et al.\textsuperscript{22} regulated the extracellular activity of the TreS enzyme (trehalose synthase) using the P\textsubscript{43} promoter to produce trehalose, a stable non-reducing disaccharide. In this study, a TreS mutant was successfully expressed and secreted in \textit{B. subtilis} WB8000.\textsuperscript{25} Currently, P\textsubscript{43} is regularly used in comparative studies to measure the strength of different promoters in \textit{B. subtilis}, showing that, although new stronger promoters can be identified, it is an important parameter for studies involving screening of promoters for the improvement of fermentation conditions and expression levels.\textsuperscript{28,29}

Several other promoters have been characterized to increase heterologous expression in \textit{B. subtilis}. These characteristics arise from the accumulation of genetic, biochemical, and structural information on the molecular mechanism underlying promoter stimulation.\textsuperscript{87} It is important to emphasize that strong promoters are necessary since their characteristics lead to more efficient transcription of the genes encoding the protein of interest. Seo and Schmidt-Dannert in 2019 used the strong promoter P\textsubscript{veg} in combination with regulatory elements of \textit{Pseudomonas putida} to investigate and control the expression of green fluorescent protein (GFP) in \textit{B. subtilis}.\textsuperscript{88} This new system has proved promising and useful for studies of metabolic engineering, synthetic biology, and production of proteins of industrial interest.\textsuperscript{88}

As a result of numerous studies, the repertoire of techniques has expanded, and improvement of the efficiency of promoters via chromosomal integration has become an option. For example, Zhou \textit{et al.}\textsuperscript{89} improved the activity of the promoter P\textsubscript{sfg} and optimized the central region -35, -10, and the upstream sequence (UP) by replacing both sequences with consensus sequences.\textsuperscript{89} The final promoter exhibited almost 26 times the activity of β-galactosidase (BgaB) and 195 times the intensity of the super-folded green fluorescent protein (sfGFP).

### The dual promoters in \textit{B. subtilis}

Another promising approach for increasing productivity that has received widespread attention due to its high efficiency and continuity is the use of dual promoters.\textsuperscript{90} In the study of Guan \textit{et al.},\textsuperscript{19} satisfactory results were achieved with the dual promoter P\textsubscript{gaiB-P\textsubscript{lipA}} which showed better performance and increased the production of aminopeptidase (AP) after 45 h of fermentation. Another dual promoter system that presented remarkable applicability was P\textsubscript{lipA-P\textsubscript{amyQ}} which produced β-cyclodextrin glycosyltransferase (β-CGTase), and mediated the expression of substantial extracellular pullulanase and the expression of x-cyclodextrin glycosyltransferase (x-CGTase).\textsuperscript{32} These enzymes were also produced using the P\textsubscript{ amyA-fuscA-amyE} triple promoter.\textsuperscript{91,92} Kang \textit{et al.}\textsuperscript{46} used a system containing a P\textsubscript{ amyE-cdd} dual promoter and a (Pac) signal peptide to increase the expression of a recombinant amidosase (Bm-Ami). The extracellular activity of Bm-Ami containing the plasmid pBSHdd2-20 reached 10.72 U/mg\textsuperscript{-1} DCW after 52 h in a scaling fermentation that, according to the authors, was the highest secretion obtained from Bm-Ami to date.\textsuperscript{46}

### The functional synthetic promoters in \textit{B. subtilis}

One technique that has proven to be useful is the development of functional synthetic promoters. Synthetic promoters are more resistant to imperfect growth conditions such as biotechnological processes that cause cellular stress or metabolic load and generate a strong transcription of target genes.\textsuperscript{93} This makes them an important alternative since natural promoters are often not universally characterized due to their poor performance.\textsuperscript{94} Han \textit{et al.}\textsuperscript{53} developed a new strategy called gradual evolution for screening robust \textit{B. subtilis} bacterial promoters that was aimed at the promoter center spacing sequence (SETarSCoP). A series of mutant promoters was obtained that overexpressed β-glucuronidase (GusA) of \textit{E. coli} and nattokinase (NK) of \textit{Bacillus natto}. The mutant promoter P\textsubscript{B134} was identified and verified as an exceptionally strong synthetic promoter. In this same study, this
The inducer-dependent promoters in B. subtilis

The first inducible expression system reported in B. subtilis was described after pioneering studies by Yansura and Henner in 1984, in which the spac promoter was induced by isopropyl-β-D-thiogalactoside (IPTG). This system is a combination of a B. subtilis phage promoter SPO-1 and the lac operator from E. coli that deactivates the constitutively produced lac repressor (LacI).

Yang et al. transformed the modified strain WB800N with the pHHT43 vector, which contained a strong promoter derived from the B. subtilis operon groE converted into an IPTG-inducible promoter. This study reported for the first time the secretion of fragments of heavy chain antibodies or nanobodies of camels (Nbs). The yields of Nbs were estimated at 15 to 20 mg from one liter of bacterial culture, which is comparable to the yields in systems for producing Nbs in E. coli (15 to 20 mg).

Scheidler et al. pioneered the development of an efficient amber suppression system in B. subtilis by using IPTG as an inducer. This system enables the expression, secretion, and direct purification of a target protein carrying non-canonical amino acids (ncAAs). The authors expressed the light chain variable domain of a murine monoclonal antibody fragment (MAK33-VL) and obtained 2 mg L⁻¹.

Other important induction systems are the ones in which induction is based on the signal peptide, such as the system LIKE (from the German "Lia-Kontrollierte Expression") that is based on the lia promoter, and the system SURE (subtilin-regulated gene expression). Both systems are strictly controlled by the addition of bacitracin and lantibiotic subtilin after the exponential phase of cell growth, thus preventing leakage of transcription in non-inductive conditions.

Studies involving the signal peptide have become increasingly viable since this mechanism can guide secretion of the heterologous protein to a specific area with more precision and thus effectively increase the solubility of the protein.

Le et al. used the robust promoter P<sub>grac212</sub>, inducible by IPTG, to overexpress human rhinovirus protease 3C (HRV3C) in the cytoplasm of B. subtilis. In the study, the proteases His-HRV3C and His-GST-HRV3C were over-expressed in the cytoplasm of B. subtilis in 11% and 16% of total cellular proteins, respectively. The largest specific protease activities were 8065 U/mg for His-HRV3C<sup>C</sup>, which indicates that it is an effective method for producing recombinant proteins in the cytoplasm of B. subtilis.

Although the IPTG inducer has been amply used, models with other chemical inducers, such as the carbohydrates sucrose, mannose, xylose, maltose, and starch, have been developed and are considered efficient and cheap. The IPTG inducer is not consumed by bacteria and this has been developed to control heterologous expression and which represent relevant engineering targets for the biotechnological production of compounds.

The inducible expression systems controlled by riboswitches in B. subtilis

As known inducers, there are a variety of synthetic riboswitches that have been developed to control heterologous expression and which represent relevant engineering targets for the biotechnological production of compounds. In this perspective, a system with great compatibility and robustness was obtained in the studies of Cui et al., in which a new genetic element composed of the promoter P<sub>43</sub> and a theophylline-riboswitch was developed and characterized in B. subtilis from the union of the constitutive expression characteristics of P<sub>43</sub> for a dose-dependent induced pattern of theophylline. The authors noted the system overexpressed β-glucuronidase with an induced expression level higher than three other strong constitutive promoters, including P<sub>sgf</sub>, P<sub>aprE</sub> and the native P<sub>43</sub>.

The conditionally activating promoters in B. subtilis

One of the simplest induction systems already described are temperature change controlled. Li et al. characterized
two temperature-sensitive promoters, P2 and P7, isolated from *B. subtilis*. The production of β-galactosidase conducted by these promoters was higher at 45°C than at 37°C. In a later study, aiming to improve the overproduction of "difficult to express" proteins, Welsch *et al.* developed a low-temperature expression system in *B. subtilis* that was based on the cold-inducible promoter of the *des* gene. The improved expression system was validated by the overproduction of xylanase, as well as an α-glucosidase from *Saccharomyces cerevisiae*, thus confirming the adequacy of this host organism for the overproduction of critical substances.

Recently, self-inducible expression systems have been attracting more attention due to the practicality of their services.53,106 Self-inducible promoters, such as P*pat* and P*cryB* can express the target gene from the late log phase to the stationary phase without the need for an inducer, which facilitates the efficient production of low-cost proteins and peptides.57,107 An inducer-independent self-inducible expression system was developed by Wenzel *et al.* using the mutant strain TQ536 to further improve cost efficiency and product yield. In this system, glucose prevented induction through repression by carbon catabolism, creating ideal conditions for self-induction. This technique led to an almost three-fold increase in production and reached a 14.6% yield of recombinant protein (eGFP).108

Guan *et al.* built a self-inducing and self-regulating expression system by simply adding glucose, using the quorum detection-related promoter (P*sfrA*). In this case, the central sequence of -10 and -35 was replaced by consensuses sequences. In the end, the system proved its efficiency by the successful production of aminopeptidase. In later studies, Guan *et al.* demonstrated that it is possible to integrate the corresponding genes downstream of the promoter in the plasmid or chromosome in the BSG1682 strain of *B. subtilis*. The results revealed that the P23 promoter, derived from the P*sfrA* Promoter, presented the best performance, and was almost twice as strong as P*sfrA*. Two heterogeneous proteins, aminopeptidase (AP) and nattokinase (NK) were overproduced in this study.109

In addition, Correa *et al.* developed a method of dynamic regulation by detecting quorum in *B. subtilis*, which was capable of self-monitoring and inducing expression without human supervision. The promoter response was 2.5 and 3.2 times stronger than the well-characterized promoters P*sfrA* and P*reg*, respectively. The researchers even applied the strongest self-induction device to produce vitamin B3 and self-induction proved to be modular and adjustable.45 Furthermore, Correa *et al.* presented a set of data to analyze different growth parameters of OD600, fluorescence measurement of GFP, luminescence, and riboflavin production. The purpose of this validation was to support the efficiency of this self-inducing device for the expression and control of genes in *B. subtilis*.110

**The inducer-free expression vectors base on pgacr**

The study of expression vectors without inducer is still a challenge due to a series of industrial production bottlenecks, but there are already some promising investigative studies.111 In this regard, Tran *et al.* generated inducer-free expression vectors using IPTG-inducible promoters in the absence of the LacI repressor and containing the strong P*grac100* promoter. The levels of expression of β-galactosidase (BgaB gene) were at least 37 times higher when compared to the inducible constructions in the absence of IPTG. These plasmids may also be convenient for studying gene expression at different scales for recombinant protein production.112

Tran *et al.* demonstrated the great potential of using strong promoters from the P*grac_2* family in the overproduction of recombinant proteins through the construction of integrative inducer-free vectors, capable of integrating into the *B. subtilis* genome in both the lacA locus and the amyE locus. In this study, the promoters P*grac_100-bgaB* and P*grac_212-bgaB* yielded 20.9% and 42% of intracellular proteins, respectively. Whereby, after the incorporation of P*grac_212-bgaB* in both the amyE and lacA loci, the expression of β-galactosidase (BgaB) was observed in up to 53.4% of intracellular proteins.

In another strategy that aims to support fundamental research studies in *B. subtilis*, Chu *et al.* constructed inducer-free expression plasmids containing the hybrid promoter P*pacr*, inducible by IPTG, suitable to express in a controlled way and at lower levels proteins with specific functions in the host cell and reporter genes. As expected, the BgaB and GFP + target genes were expressed at low levels compared to inducer-free plasmids with the strong promoters P*grac100* and P*grac100_2* about 16.2 to 20.3 times for BgaB and 24.7 to 34.3 times for GFP +, respectively. The authors concluded that the use of these inducer-free vectors containing the P*pacr* promoter enabled the constitutive expression of heterologous recombinant proteins at low levels desired in *B. subtilis*.114

**Conclusions**

In summary, the literature consulted expands the knowledge regarding the functional expression systems that are commercially available and developed from different lineages of *B. subtilis*. We note the existence of a large arsenal of precursors and recent studies that accredit this bacterium as one of the main platforms for the expression of heterologous proteins, thus promoting progress in bioproduction. Furthermore, its wide use is justified due to its genetic and metabolic characteristics that allow proper planning and provide experimental security. The success of this model in expressing biocomposites is due to the development of massive strategies aimed at creating and constantly guaranteeing the efficiency of its promoters, plasmids, the use of low-cost varied inducers, and transcriptional self-regulatory elements, among others. As a result, the high technological status achieved increasingly encourages the improvement of existing genetic engineering techniques, as well as the search for the development of new efficient approaches, which makes the Gram-positive *B. subtilis* bacteria a robust and promising host with biotechnological excellence for the optimal expression of different recombinant proteins with valuable applications. Although the
advances in omics studies are clear and decisive for the affirmation of this robust biotechnological host, and many strategies have culminated in the expression of various recombinant proteins, future studies need to focus on overcoming many limitations regarding the understanding and exploration of secretion pathways. New strategies must need to be developed to overcome current bottlenecks, e.g. expression of medical and industrial importance multiple subunits proteins.

AUTHORS’ CONTRIBUTIONS

All authors participated in the design and review of the manuscript. CCS, LAMM, and SDSVP performed the literature review, CCS wrote the manuscript, and JMG provided the images.

ACKNOWLEDGMENTS

We thank the Universidade Federal do Amazonas (UFAM), Instituto Leônidas e Maria Deane (ILMD), Fundação Oswaldo Cruz (FIOCRUZ AMAZÔNIA) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for their support.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iDs

Caio Coutinho de Souza https://orcid.org/0000-0002-5107-8727
Soraya dos Santos Pereira https://orcid.org/0000-0003-2116-7670

REFERENCES

1. Liu Y, Liu L, Li J, Du G, Chen J. Synthetic biology toolbox and chassis development in Bacillus subtilis. Trends Biotechnol 2019;37:548–62
2. Shumann W. Productions of recombinant proteins in Bacillus subtilis. Adv Appl Microbiol 2007;62:137–89
3. Beauregard PB, Chai Y, Vlamakis H, Losick R, Kolter R. Bacillus subtilis biofilm induction by plant polysaccharides. Proc Natl Acad Sci U S A 2013;110:E1621–30
4. Fukushima T, Szurmant H, Kim EJ, Perego M, Hoch JA. A sensor histidine kinase co-ordinates cell wall architecture with cell division in Bacillus subtilis. Mol Microbiol 2008;69:621–32
5. Vlamakis H, Chai Y, Beauregard PB, Losick R, Kolter R. Sticking together: building a biofilm the Bacillus subtilis way. Nat Rev Microbiol 2013;11:157–68
6. Harwood CR, Mouillon JM, Pohl S, Arnau J. Secondary metabolite production and the safety of industrially important members of the Bacillus subtilis group. FEMS Microbiol Rev 2018;42:721–38
7. Rosales-Mendoza S, Angulo C. Bacillus subtilis comes of age as a vaccine production host and delivery vehicle. Expert Rev Vaccines 2015;14:1135–48
8. Heravi KM, Watzlawick H, Altenbuchner J. Development of an anhydroractylcycline-inducible expression system for expression of a neopullullanase in B. subtilis. Plasmid 2015;82:35–42
9. Phan T, Huynh P, Truong T, Nguyen H. A generic protocol for intracellular expression of recombinant proteins in Bacillus subtilis. Methods Mol Biol 2017;1586:325–34
10. Hanif MU, Gul R, Hanif M, Hashmi AA. Heterologous secretory expression and characterization of dimerized bone morphogenetic protein 2 in Bacillus subtilis. BioMed Res Int 2017. DOI: 10.1155/2017/935037
11. Radeck J, Kraft K, Bartels J, Cikovic T, Durr F, Emenegger J, Kelterborn S, Sauer C, Fritz G, Gebbard S, Mascher T. The bacillus BioBrick box: generation and evaluation of essential genetic building blocks for standardized work with Bacillus subtilis. J Biol Eng 2013;7:1–17
12. Popp PF, Dotzler M, Radeck J, Bartels J, Mascher T. The bacillus BioBrick box 2.0: expanding the genetic toolbox for the standardized work with Bacillus subtilis. Sci Rep 2017;7:1–3
13. Karpov DS, Domashin AI, Kotlov MI, Osipova PG, Kiseleva SV, Seregina TA, Goncharenko AV, Mironov AS, Karpov VL, Poddubko SV. Biotechnological potential of the Bacillus subtilis 20 strain. Mol Biol 2020;54:119–27
14. Chen J, Zhao L, Fu G, Zhou W, Sun Y, Zheng P, Sun J, Zhang D. A novel strategy for protein production using non-classical secretion pathway in Bacillus subtilis. Microb Cell Fact 2016;15:1–16
15. Cui W, Han L, Suo F, Liu Z, Zhou L, Zhou Z. Exploitation of Bacillus subtilis as a robust workhorse for production of heterologous proteins and beyond. World J Microbiol Biotechnol 2018;34:1–19
16. Harwood CR, Bacillus subtilis and its relatives: molecular biological and industrial workhorses. Trends Biotechnol 1992;10:247–56
17. Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni GO, Azevedo V, Borris R. The complete genome sequence of the Gram-positive bacterium Bacillus subtilis. Nature 1997;390:249–56
18. Barbe V, Cruveiller S, Kunst F, Lenoble P, Meurice G, Sekowska A, Vallenet D, Wang T, Moszer I, Médigue C, Danchin A. From a consensus sequence to a unified sequence: the Bacillus subtilis 168 reference genome a decade later. Microbiology 2009;155:1758–75
19. Burkholder PR, Giles NH. Induced biochemical mutations in Bacillus subtilis. Am J Bot 1947;34:345–8
20. Zhang K, Su L, Wu J. Recent advances in recombinant protein production by Bacillus subtilis. Annu Rev Food Sci Technol 2020;11:295–318
21. Terpe K. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 2006;72:211–22
22. Xiang M, Kang Q, Zhang D. Advances on systems metabolic engineering of Bacillus subtilis as a chassis cell. Synth Syst Biotechnol 2020;5:245–51
23. Calero P, Nikol PI. Chasing bacterial chassis for metabolic engineering: a perspective review from classical to non-traditional microorganisms. Microb Biotechnol 2019;12:98–124
24. Yang T, Irene K, Liu H, Liu S, Zhang X, Xu M, Rao Z. Enhanced extracellular gamma glutamyl transeptidase production by overexpressing of PrsA lipoproteins and improving its mRNA stability in Bacillus subtilis and application in biosynthesis of L-theanine. J Biotechnol 2019;302:85–91
25. Schallmey M, Singh A, Ward OP. Developments in the use of bacillus species for industrial production. Curr J Microbiol 2004;50:1–17
26. Hirooka K, Tamano A. Bacillus subtilis highly efficient protein expression systems that are chromosomally integrated and controllable by glucose and rhamnose. Biosci Biotechnol Biochem 2018;82:1942–54
27. Liu H, Wang S, Song L, Yuan H, Liu K, Meng W, Wang T. Trehalose production using recombinant trehalose synthase in Bacillus subtilis by integrating fermentation and biocatalysis. J Agric Food Chem 2019;67:9314–24
28. Wang Z, Li X, Tian J, Chu Y, Tian Y. Cloning, heterologous expression and characterization of a novel streptomycys trypsin in Bacillus subtilis SCKs. Int J Biol Macromol 2019;147:890–7
29. Zhao X, Xu J, Tan M, Zhen J, Ma Y, Zheng HC, Song H. High copy number and highly stable Escherichia coli-Bacillus subtilis shuttle plasmids based on pWB900. Microb Cell Fact 2020;19:1–12
30. Feng Y, Liu S, Jiao Y, Wang Y, Wang M, Du G. Gene cloning and expression of the l-asparaginase from Bacillus cereus BDRD-ST26 in Bacillus subtilis WB600. J Biosci Bioeng 2019;127:418–24

31. Song W, Nie Y, Mu QX, Xu Y. Enhancement of extracellular expression of Bacillus nagenonensis pullulanase from recombinant Bacillus subtilis: effects of promoter and host. Protein Expr Purif 2016;124:23–31

32. Mu D, Lu J, Qiao M, Kuipers OP, Zha J, Li X, Yang P, Zhao Y, Luo S, Wu X, Jiang S, Zheng Z. Heterologous signal peptides-directing secretion of Streptomyces mobaraensis transglutaminase by Bacillus subtilis. Appl Microbiol Biotechnol 2018;102:5533–43

33. Yu X, Xu J, Liu X, Chu X, Wang P, Tian J, Wu N, Fan Y. Identification of a highly efficient stationary phase promoter in Bacillus subtilis. Sci Rep 2015;5:1–9

34. Guan C, Cui W, Cheng J, Zhou L, Guo J, Hu X, Xial G, Zhou Z. Construction and development of an auto-regulatory gene expression system in Bacillus subtilis. Microb Cell Fact 2015;14:1–15

35. Chen J, Gai Y, Fu G, Zhou W, Zhang D, Wen J. Enhanced extracellular production of alpha-amylase in Bacillus subtilis by optimization of regulatory elements and over-expression of PrsA lipoprotein. Biotechnol Lett 2015;37:899–906

36. He W, Mu W, Jiang B, Yan X, Zhang T. Construction of a food grade recombinant Bacillus subtilis based on replicative plasmids with an auxotrophic marker for biotransformation of d-fructose to d-allulose. J Agric Food Chem 2016;64:3243–50

37. 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;16:1–15

38. Phan TT, Tran LT, Schumann W, Nguyen HD. Development of Pgrac100-based expression vectors allowing high protein production levels in Bacillus subtilis and relatively low basal expression in Escherichia coli. Microb Cell Fact 2015;14:1–9

39. Deng Y, Nie Y, Zhang Y, Wang Y, Xu Y. Improved inducible expression of Bacillus nagenonensis pullulanase from recombinant Bacillus subtilis by enhancer regulation. Protein Expr Purif 2018;148:9–15

40. Song Y, Fu G, Dong H, Li J, Du Y, Zhang D. High-efficiency secretion of β-mannanase in Bacillus subtilis through protein synthesis and secretion optimization. J Agric Food Chem 2017;65:2540–8

41. Ma RJ, Wang YH, Liu L, Bai LL, Ban R. Production enhancement of the extracellular lipase LipA in Bacillus subtilis: effects of expression system and sec pathway components. Protein Expr Purif 2018;142:81–7

42. Chen J, Fu G, Gai Y, Zheng P, Zhang D, Wen J. Combinatorial sec pathway analysis for improved heterologous protein secretion in Bacillus subtilis: identification of bottlenecks by systematic gene over-expression. Microb Cell Fact 2015;14:1–15

43. Su HH, Chen JC, Chen PT. Production of recombinant human epidermal growth factor in Bacillus subtilis. J Taiwan Inst Chem En 2020;106:86–91

44. Halmenschlag B, Putri SP, Fukusaki E, Blank LM. Poly-γ-glutamic acid production by Bacillus subtilis 168 using glucose as the sole carbon source: a metabolomic analysis. J Biosci Bioeng 2020;120:727–82

45. Correa GG, Lins M, Silva BF, Paiva GB, Zocca VF, Ribeiro NV, Picheli FP, Mack M, Pedrolli DB. A modular autoinduction device for control of gene expression in Bacillus subtilis. Metab Eng 2020;61:326–34

46. Kang XM, Cai X, Huang ZH, Liu QZ, Zheng YG. Construction of a highly active secretory expression system in Bacillus subtilis of a recombinant amidase by promoter and signal peptide engineering. Int J Biol Macromol 2020;153:833–41

47. Zhang L, Li G, Zhan N, Sun T, Cheng B, Li Y, Shan A. Expression of a Pseudomonas aeruginosa-targeted antimicrobial peptide TW9 in Bacillus subtilis using a maltose-inducible vector. Process Biochem 2019;81:22–7

48. Han L, Suo F, Jiang C, Gu J, Li N, Zhang N, Zhou Z. Fabrication and characterization of a robust and strong bacterial promoter from a semi-rationally engineered promoter library in Bacillus subtilis. Process Biochem 2017;61:56–62

49. 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. N Biotechnol 2016;33:372–9

50. Cui W, Han L, Cheng J, Liu Z, Zhou L, Guo J, Zhou Z. Engineering an inducible gene expression system for Bacillus subtilis from a strong constitutive promoter and a theophylline-activated synthetic riboswitch. Microb Cell Fact 2016;15:1–13

51. Heinrich J, Drewniok C, Neugebauer E, Kellner H, Wiegert T. The YaoA signal peptide directs efficient secretion of different heterologous proteins fused to a StrepII-SUMO tag in Bacillus subtilis. Microb Cell Fact 2019;18:2–14

52. Yang M, Zhu G, Korza G, Sun X, Setlow P, Li J. Engineering Bacillus subtilis as a versatile and stable platform for production of nanobodies. Appl Environ Microbiol 2020;86:1–15

53. Schieder CM, Vrabel M, Schneider S. Genetic code expansion, protein expression, and protein functionalization in Bacillus subtilis. ACS Synth Biol 2020;9:486–93

54. Zhang L, Li X, Zhan N, Sun T, Li J, Shan A. Maltose induced expression of cepacrin AD by SUMO technology in Bacillus subtilis WB800N. Protein J 2020;39:383–91

55. Zhang L, Wei D, Zhan N, Sun T, Shan B, Shan A. Heterologous expression of the novel z-helical hybrid peptide PR-FO in Bacillus subtilis. Bioprocess Biosyst Eng 2020;43:1619–27

56. Fu L, Wang Y, Ju J, Cheng L, Xu Y, Yu B, Wang L. Extracellular production of active-form Streptomyces mobaraensis transglutaminase in Bacillus subtilis. Appl Microbiol Biotechnol 2020;104:623–31

57. Sun W, Yu Y, Ding W, Wang L, Wu L, Liu L, Che Z, Zhu L, Liu Y, Chen X. An auto-inducible expression and high cell density fermentation of beefy meaty peptide with Bacillus subtilis. Bioprocess Biosyst Eng 2020;43:701–10

58. Pang B, Zhou L, Cui W, Liu Z, Zhou Z. Production of a thermostable pullulanase in Bacillus subtilis by optimization of the expression elements. Starch 2020;72:11–12

59. Promchai R, Promdonkpit B, Tanapongpipat S, Vissessanguan W, Eurwilairach L, Luxanani P. A novel salt-inducible vector for efficient expression and secretion of heterologous proteins in Bacillus subtilis. J Biotechnol 2016;222:86–93

60. Ramos KRM, Valdehuesa KNG, Cabulong RB, Moron LS, Nisola GM, Hong SK, Chung WJ. Overexpression and secretion of AgaA7 from pseudalteromonas hodoensis sp. nov in Bacillus subtilis for the depolymerization of agarose. Enzyme Microb Technol 2016;90:19–25

61. Le VD, Phan TTP, Nguyen TM, Brunsved L, Schumann W, Nguyen HD. Using the IPTG-inducible Praft212 promoter for overexpression of human rhinovirus 3C protease fusions in the cytoplasm of Bacillus subtilis cells. Curr Microbiol 2019;76:1477–86

62. Tao Z, Fu G, Wang S, Jin Z, Wen J, Zhang D. Hyper-secretion mechanism exploration of a heterologous creatinase in Bacillus subtilis. Biochem Eng J 2019. DOI: 10.1016/j.bj.2019.107419

63. Han L, Cui W, Suo F, Miao S, Hao H, Chen Q, Guo J, Liu Z, Zhou L, Zhou Z. Development of a novel strategy for robust synthetic bacterial promoters based on a stepwise evolution targeting the spacer region of the core promoter in Bacillus subtilis. Microbiol Cell Fact 2019;18:2–14

64. Liu Y, Shi C, Li D, Chen X, Li J, Zhang Y. Engineering a highly efficient expression system to produce BcaPRO protease in Bacillus subtilis by an optimized promoter and signal peptide. Int J Biol Macromol 2019;138:903–11

65. Zhao L, Chen J, Sun J, Zhang D. Multimer recognition and secretion by the non-classical secretion pathway in Bacillus subtilis. Sci Rep 2017;7:1–18

66. Wang M, Wu J, Wu D. Cloning and expression of the sucrase phosphorylase gene in Bacillus subtilis and synthesis of kojibiose using the recombinant enzyme. Microb Cell Fact 2018;17:1–9

67. Xi X, Ni K, Hao H, Shang Y, Zhao B, Qian Z. Secretory expression in Bacillus subtilis and biochemical characterization of a highly thermo-stable polyethylene terephthalate hydrolase from bacterium HR29. Enzyme Microbial Technol 2021. DOI: 10.1016/j.enzmictec.2020.109715

68. Kanaya S, Yamada Y, Kudo Y, Ikemura T. Studies of codon usage and tRNA genes of 18 unicellular organisms and quantification of Bacillus subtilis tRNAs: gene expression level and species-specific diversity of codon usage based on multivariate analysis. Gene 1999;238:143–55

69. Moszer I, Rocha EF, Danchin A. Codon usage and lateral gene transfer in Bacillus subtilis. Curr Opin Microbiol 1999;2:524–8
70. Tjalsma H, Antelmann H, Jongbloed JD, Braun PG, Darmon E, Dorenbos R, Dubois JY, Westers H, Zanen G, Quax WJ, Kuipers OP, Bron S, Hecker M, Van Dijl JM. Proteomics of protein secretion by Bacillus subtilis: separating the “secrets” of the secretome. Microbiol Mol Biol Rev 2004;68:207–33

71. Frain KM, Robinson C, Van Dijl JM. Transport of folded proteins by the Tat system. Protein J 2019;38:377–88

72. Molière N, Turgay K. Chaperone-protease systems in regulation and protein quality control in Bacillus subtilis. Res Microbiol 2009;160:637–44

73. Jongbloed JD, Antelmann H, Hecker M, Niijland R, Bron S, Airaksinen U, Braun PG. Selective contribution of the twin-arginine translocation pathway to protein secretion in Bacillus subtilis. J Biol Chem 2002;277:44068–78

74. Neef J, Bongiorni C, Schmidt B, Goosens VJ, Van Dijl JM. Relative contributions of non-essential sec pathway components and cell envelope-associated proteases to high-level enzyme secretion by Bacillus subtilis. Microb Cell Fact 2020;19:1–13

75. Tjalsma H, Bolhuis A, Jongbloed JDH, Bron S, Van Dijl JM. Signal peptide-dependent protein transport in Bacillus subtilis: a genome-based survey of the secretome. Microbiol Mol Biol Rev 2000;64:515–47

76. Hirose I, Sano K, Shioda I, Kumanoo M, Nakamura K, Yamane K. Proteome analysis of Bacillus subtilis extracellular proteins: a two-dimensional protein electrophoretic study the SWISS-PROT accession numbers for the N-terminal amino acid sequences reported in this paper are: P00891 for AmyE; P54507 for CotN; O07921 for ctn; P09124 for gap; P26901 for kata; P39116 for pel; P39824 for ponP; P54375 for sodA; P29141 for vpr; Q07833 for wasA; P54423 for WprA; P54327 for XdG; Q54701 for yndD; P94421 for yDQ; O31803 for ycmM; O05512 for YdhT; O34952 for YfIE; O06487 for YfIf; O31737 for YlqB; P96740 for YWdI; P24110 for YxaK; P94356 for YxkC. Microbiology 2000;146:65–75

77. Fu LL, Xu ZR, Li WF, Shuai JB, Lu P, Hu CX. Protein secretion pathways in Bacillus subtilis: implication for optimization of heterologous protein secretion. Biotechnol Adv 2007;25:1–12

78. Oguro A, Kakeshiba H, Honda K, Takamatsu H, Nakamura K, Yamane K, Srb: a Bacillus subtilis gene encoding a homolog of the alpha-subunit of the mammalian signal recognition particle receptor. DNA Res 1995;2:95–100

79. Wahlström E, Viti-Kainen M, Kontinen VP, Sarvas M. The extracytoplasmic folding factor PrsA is required for protein secretion only in the presence of the cell wall in Bacillus subtilis. Microbiology 1999;145:569–57

80. Schiebel E, Driessen AJ, Hartl FU, Wickner W. Delta mu H. Mol Cell 2003;14:967–80

81. DeLisa MP, Tullman D, Georgiou G. Folding quality control in the secretome. Microbiol Mol Biol Rev 2004;68:207–33

82. Wahlström E, Viti-Kainen M, Kontinen VP, Sarvas M. The extracytoplasmic folding factor PrsA is required for protein secretion only in the presence of the cell wall in Bacillus subtilis. Microbiology 1999;145:569–57

83. Wang PZ, Doi RH. Overlapping promoters transcribed by Bacillus subtilis sigma 55 and sigma 37 RNA polymerase holoenzymes during growth and stationary phases. J Bacteriol 1984;164:819–25

84. Song BF, Neuhard J. Chromosomal location, cloning and nucleotide sequence of the Bacillus subtilis cdd gene encoding cytidine/deoxycytidine deaminase. Mol Gen Genet MGG 1989;216:462–8

85. Zhang XZ, Cui ZL, Hong Q, Li SP. High-level expression and secretion of methyl parathion hydrolyse in Bacillus subtilis wild type. Appl Environ Microbiol 2005;71:4101–3

86. Xia Y, Chen W, Zhao J, Tian F, Zhang H, Ding X. Construction of a new food-grade expression system for Bacillus subtilis based on theta replication plasmids and auxotrophic complementation. Appl Microbiol Biotechnol 2007;76:643–50

87. Meijer WJ, Salas M. Relevance of up elements for three strong Bacillus subtilis phage 432 promoters. Nucleic Acids Res 2004;32:1166–76

88. Seco SO, Schmidt-Dannert C. Development of a synthetic cumate-inducible gene expression system for bacillus. Appl Microbiol Biotechnol 2019;103:303–13

89. Zhou C, Ye B, Cheng S, Zhao L, Liu Y, Jian J, Yan X. Promoter engineering enables overproduction of foreign proteins from a single copy expression cassette in Bacillus subtilis. Microb Cell Fact 2019;18:1–11

90. Rao Y, Cai D, Wang H, Xu Y, Xiong S, Gao L, Ma X. Construction and application of a dual promoter system for efficient protein production and metabolic pathway enhancement in Bacilluslicheniformis, J Biotechnol 2020;312:1–10

91. Liu X, Wang H, Wang B, Pan L. Efficient production of extracellular pullulanase in Bacillus subtilis ATCC6051 using the host strain construction and promoter optimization expression system. Microb Cell Fact 2018;17:1–12

92. Meng F, Zhu X, Nie T, Lu J, Bie X, Lu Y, Lu Z. Enhanced expression of pullulanase in Bacillus subtilis by new strong promoters mined from transcriptome data, both alone and in combination. Front Microbiol 2018. DOI: 10.3389/fmicb.2018.02635

93. Rajkumar AS, Liu G, Bergenholt D, Arsovsk A, Kristensen M, Nielsen J, Jensen MK, Keasling JD. Engineering of synthetic, stress-responsive yeast promoters. Nucleic Acids Res 2016;44:e136–e136

94. Wang Y, Liu Q, Weng H, Shi Y, Chen J, Du G, Kang Z. Construction of synthetic promoters by assembling the sigma factor binding –35 and –10 boxes. Biotechnol J 2019. DOI: 10.1002/biot.201800298

95. Yansura DG, Henner DJ. Use of the Escherichia coli lac repressor and operator to control gene expression in Bacillus subtilis. Proc Natl Acad Sci U S A 1984;81:439–43

96. Salema V, Fernandez LA. High yield purification of nanobodies from the periplasm of Escherichia coli as fusions with the maltose binding protein. Protein Expr Purif 2013;92:42–8

97. Ming YM, Wei ZW, Lin CY, Sheng GY. Development of a Bacillus subtilis expression system using the improved pgI promoter. Microb Cell Fact 2010;9:1–8

98. Sun T, Altenbuchner J. Characterization of a mannone utilization system in Bacillus subtilis. J Bacterial 2010;192:2128–39

99. Heravi KM, Wenzel M, Altenbuchner J. Regulation of mtl operon promoter of Bacillus subtilis: requirements of its use in expression vectors. Microb Cell Factories 2011;10:1–19

100. Toymentseva AA, Schrecke K, Sharipova MR, Mascher T. The like system, a novel protein expression toolbox for Bacillus subtilis based on the lal promoter. Microb Cell Fact 2012;11:1–13

101. 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;36:1783–9

102. Desai SK, Gallivan JP. Genetic screens and selections for small molecules based on a synthetic riboswitch that activates protein translation. J Am Chem Soc 2004;126:13247–54

103. Suez B, Fink B, Berens C, Stentz R, Hillen W. A th fortified responsive riboswitch based on helix slipping controls gene expression in vivo. Nucleic Acids Res 2004;32:1610–4

104. Li W, Li LX, Li SY, Li S, Gong YS, Yang MM, Chen YL. Characterization of two temperature-inducible promoters recently isolated from B. subtilis. Biochem Biophys Res Commun 2007;358:1148–53

105. Welsch N, Homuth G, Schweder T. Stepwise optimization of a low-temperature Bacillus subtilis expression system for “difficult to express” proteins. Appl Microbiol Biotechnol 2015;99:6363–76

106. Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Bathe TS, Redding-Johann AM, Petzold Mukhopadhyay CJ, Lee TS, Adams PD, Keasling JD. Engineering dynamic pathway regulation using stress-response promoters. Nat Biotechnol 2013;31:1039–46

107. Lee SJ, Pan JG, Park SH, Choi SK. Development of a stationary phase-specific autoinducible expression system in Bacillus subtilis. J Biotechnol 2010;149:16–20

108. Wenzel M, Muller A, Siemann-Herzberg M, Altenbuchner J. Self-inducible Bacillus subtilis expression system for reliable and
inexpensive protein production by high-cell-density fermentation. 
*Appl Environ Microbiol* 2011;77:6419–25

109. Guan C, Cui W, Cheng J, Zhou L, Liu Z, Zhou Z. Development of an efficient autoinducible expression system by promoter engineering in *Bacillus subtilis*. *Microb Cell Fact* 2016;15:1–12

110. Correa GG, Lins Mrdcr Silva BF, Paiva GB, Zocca VFB, Ribeiro NV, Picheli FP, Mack M, Pedrolli DB. Dataset for supporting a modular autoinduction device for control of gene expression in *Bacillus subtilis*. *Data Brief* 2020;31:105736

111. Liu H, Wang X, Yang S, Wang R, Wang T. Saturation mutagenesis and self-inducible expression of trehalose synthase in *Bacillus subtilis*. *Biotechnol Prog* 2019. DOI: 10.1002/btpr.2826

112. Tran DTM, Phan TTP, Huynh TK, Dang NTK, Huynh PTK, Nguyen TM, Truong TTT, Tran TL, Schumann S, Nguyen HD. Development of inducer-free expression plasmids based on IPTG-inducible promoters for *Bacillus subtilis*. *Microb Cell Fact* 2017;16:1–10

113. Tran DTM, Phan TTP, Doan TTN, Tran TL, Schumann W, Nguyen HD. Integrative expression vectors with *pgrac* promoters for inducer-free overproduction of recombinant proteins in *Bacillus subtilis*. *Biotechnol Rep* 2020. DOI: 10.1016/j.btre.2020.e00540

114. Chu PTB, Phan HTT, Nguyen HD, Phan TTP. Development of inducer-free expression plasmids using IPTG-inducible *pgrac* promoter for *Bacillus subtilis*. *VJSTE* 2021;63:64–70