Cloning Systems in *Bacillus*: Bioengineering of Metabolic Pathways for Valuable Recombinant Products

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Abstract: Representatives of the genus *Bacillus* have been established as one of the most important industrial microorganisms in the last few decades. Genetically modified *B. subtilis* and, to a lesser extent, *B. licheniformis*, *B. amyloliquefaciens*, and *B. megaterium* have been used for the heterologous expression of numerous proteins (enzymes, vaccine components, growth factors), platform chemicals, and other organic compounds of industrial importance. Vectors designed to work in *Bacillus* spp. have dramatically increased in number and complexity. Today, they provide opportunities for genetic manipulation on every level, from point mutations to systems biology, that were impossible even ten years ago. The present review aims to describe concisely the latest developments in the shuttle, integrative, and CRISPR-Cas9 vectors in *Bacillus* spp. as well as their application for large-scale bioengineering with the prospect of producing valuable compounds on an industrial scale. Genetic manipulations of promoters and vectors, together with their impact on secretory and metabolic pathways, are discussed in detail.

Keywords: *Bacillus subtilis*; *B. licheniformis*; *B. amyloliquefaciens*; *B. megaterium*; heterologous expression; signal peptides; promoters; vectors; metabolic engineering; CRISPR/Cas9

1. Introduction

The genus *Bacillus* represents a diverse group of Gram-positive, endospore-forming bacterial species with the well-deserved fame of being potent, versatile, and one of the most promising industrial microorganisms yet discovered. They have an average genome size between 3.4 and 6.0 Mbp [1] and a low GC% content ranging from ~35% in *B. thuringiensis* to 43.5–46.4% in *B. subtilis*, *B. licheniformis*, and *B. velezensis* [2–5]. The distinct advantages of *Bacillus* spp. used as microbial cell factories include a short fermentation cycle (around 48 h), ease of cultivation, and robust growth; non-pathogenic Generally Regarded as Safe (GRAS) status; the ability to secrete recombinant proteins in the medium; and the lack of external and endotoxins [1,6]. In recent decades, genetically modified *B. subtilis* and other *Bacillus* spp., notably *B. licheniformis*, *B. amyloliquefaciens*, and *B. megaterium*, have been used prodigiously for the heterologous production of anything from pharmaceutical proteins (antibody fragments, interferons and interleukins, growth factors, hormone precursors, and antimicrobial peptides) to industrial and food-grade enzymes. Large-scale genetic engineering has made possible the redirection of whole metabolic pathways toward valuable non-protein products such as organic acids, alcohols, and vitamins. Compared to *Escherichia coli*, its chief rival among recombinant bacteria [7], *Bacillus* spp. used to have some limitations in the past associated with the relatively smaller number of suitable regulatory regions for gene expression, peculiarities in the secretion of recombinant proteins, and the need for the selection of domesticated host strains [8]. This situation has been rapidly changed with the development of increasingly diverse and versatile vectors, novel genome editing tools such as the CRISPR/Cas9 system, and the construction of convenient strains...
deficient in multiple proteases. As numerous biotechnological applications of recombinant Bacillus strains have accumulated in recent years, the present work aims to summarize the state of the art in the progress of genetic engineering in Bacilli, with a focus on the specific practical applications of obtaining industrially important products. New strategies in strain and vector construction, metabolic engineering approaches, genome editing tools, and the complex synthetic biology perspective are discussed below.

2. Cloning Systems in Bacillus spp.

2.1. Host Strains

*B. subtilis* is by far the most widely used *Bacillus* species in biotechnology. Named *Bacillus subtilis* (“the subtle rod”) by Ferdinand Julius Cohn in 1872, it has become the most studied and best understood bacterial organism after *E. coli*, and rightly so, it has been called “a Swiss army knife of science and biotechnology”. In addition to its numerous applications in biotechnology, *B. subtilis* is useful in some unexpected areas, such as filling concrete cracks [9,10]. *B. subtilis* 168, the most popular strain in biotechnology, was obtained from *B. subtilis* Marburg through X-ray induction back in 1947. Several protease-deficient strains have been derived from it, notably WB600, WB700, and WB800 [11]. The lack of proteases is a great advantage when a protein of interest is to be expressed. *B. subtilis* WB800, deficient in all eight extracellular proteases (NprE, AprE, Epr, Bpr, Mpr, NprB, Vpr, and WprA), is commercially available and has had its genome fully sequenced [12].

*B. amyloliquefaciens*, a metabolically robust bacterium, has been employed in various feats of metabolic engineering, such as the synthesis of the essential non-protein amino acid ornithine from inulin [13,14]. *B. licheniformis* has emerged as the most promising natural producer of 2,3-butanediol. Many strains have been subjected to media optimization and genetic engineering to improve their native production [15,16]. *B. megaterium* is the undisputed leader in the bioproduction of Vitamin B12, also known as cobalamin [17].

Every *Bacillus* sp. is a unique organism with highly specific composition and functions. However, certain parts of their molecular machinery appear to be interchangeable. A large study of nearly 400 signal peptides from *B. subtilis* and *B. licheniformis*, using subtilisin as a target protein, found that some of the most efficient among them work equally well in both species, although this efficiency cannot be predicted *a priori* [18].

Since natural antibiotic resistance is characteristic of many *Bacillus* spp., it needs to be taken into account during the selection of positive clones. For instance, *B. subtilis* and *B. licheniformis* are resistant to macrolide antibiotics (erythromycin) due to the presence of *ermD* and *ermK* genes responsible for the post-transcriptional methylation of the 23S bacterial ribosomal RNA [19]. Almost all bacilli are resistant to streptomycin (which acts as a protein synthesis inhibitor) and also to cell-wall synthesis inhibitor ampicillin—up to 2048 µg/mL [20]. However, *B. subtilis* and other bacilli are susceptible to other popular antibiotics used in molecular cloning, such as kanamycin, tetracycline, vancomycin, and gentamicin. Sensitivity to chloramphenicol is a species-specific feature that is often used as a positive selection marker [19]. However, there is no universal rule for predicting the antibiotic resistance profile of *Bacillus* sp. host strains, and the applicability of certain antibiotics for clone selection must be verified experimentally.

2.2. Vectors

*Bacillus* vectors, similarly to those applied in *E. coli*, contain several required genetic regions: one or more promoters (P), regulatory regions (such as the classic lac operon, for instance), specific sites from which replication occurs (origins, ori), and one or more genes allowing selection of recombinants (usually those that confer antibiotic resistance). A somewhat unusual feature, frequently if not always present, is the signal peptide (SP), which encodes a short chain of 20–30 amino acids critical for the export of the desired protein into the extracellular space. Vectors used in *Bacillus* spp. may be said to be of two major types: autonomously replicating and integrative.
2.2.1. Autonomously Replicating Vectors

Depending on their composition, autonomously replicating vectors can be cloned or expressed, with the latter containing all necessary regulatory elements for gene expression and protein synthesis and processing. The vectors used for heterologous expression of intracellular and extracellular proteins in *Bacillus* spp. are usually shuttle vectors with the respective origins of replication for *E. coli* and *Bacillus* spp. Many shuttle vectors are commercially available and widely used. One of the most popular is pMA5 (Figure 1), derived from pUC110 and widely used for the production of human interleukin-3 [21] and human recombinant fibroblast growth factor 21 [22].

![Figure 1](image_url)

Figure 1. Selected vectors used in recombinant *Bacillus* spp. (a) pBacTag-DYKDDDK; (b) pHY300PLK; (c) PHYcas9dsr; (d) pMa5. Designations: KanR, aminoglycoside phosphotransferase gene; TcR, tetracycline efflux protein gene; AmpR, β-lactamase; ori, high-copy-number ColE1/pMB1/pBR322/pUC origin of replication; ColE1, *E. coli* origin of replication; p15A ori, the origin that can be propagated by *E. coli* containing ColE1 origin plasmid; f1 ori, f1 bacteriophage origin of replication; repB, encoding RepB replication protein; CAP binding site, *E. coli* catabolite activator protein; lacI, Lac Inhibitor protein gene; fd terminator, central terminator from bacteriophage fd; cat promoter, promoter of the *E. coli* cat gene en-coding chloramphenicol acetyltransferase; T1T2T0, encodes the Lambda T0 and rnmB T1, T2 terminators; HpaII, constitutive promoter; AmpR promoter, promoter of AmpR gene; Pspac, IPTG-inducible promoter; TtrpA, terminator sequence of the trpA gene; Flag, Flag® tag; PamyQ, promoter of the *B. amyloliquefaciens* α-amylase gene; Cas9, dual RNA guided endonuclease; sgRNA, single guide RNA of Cas9.
Another shuttle vector lately fashionable is pBE-S, successfully used for the secretion of thermostable α-glucosidase by *B. subtilis* RIK1285 [23].

Shuttle vectors can be derived from various sources, but in all cases, it is crucial to establish their segregational stability (or lack thereof). One study screened 55 *B. subtilis* isolates from various natural regions in Belarus, identified large plasmids in 20% of them, and constructed a new shuttle vector based on a theta replicon without homolog in the databases. The new vector had a low copy number (6 copies per chromosome) but was stably inherited—less than 10% of the cells lost the plasmid after 20 generations [24]. New expression vectors based on pMTLBS72 were tested with several different promoters (including P_ghiB, which can be induced by heat and acid shock and by ethanol) and htpG (a heat shock gene) as a reporter in *B. subtilis* 1012. One (pHCMC04) remained fully stable for 100 generations, while another (pHCMC05) suffered segregational instability: after 60 generations, about 60% of the cells lost the plasmid [25].

Stable shuttle vectors were developed for *B. thuringiensis* strains to increase their insecticidal activity due to the so-called crystal toxins (encoded by cry genes) typically encoded by large natural plasmids. Examples of such vectors include pHT3101, pHT315, pHBLBIV, and pEMB0557, all of them with two origins of replication: for Gram-negative bacteria (e.g., ori pUC18, ori pBeloBAC11) and for Gram-positive bacteria (e.g., ori pHT1030, ori pBLB, ori p60), both named after the original vectors from which they were derived [26].

Expression vectors that are not shuttle vectors are relatively rare, for the simple reason that it is easier to obtain them in sufficient amounts in *E. coli* before putting them to work in *Bacillus* spp. But they do exist and seem to work. Two of them were apparently developed for *B. subtilis*, one for the expression of intracellular proteins (pNDH33) and one for the expression of extracellular proteins (pNDH37, with an amyQ signal peptide). Both had the IPTG-inducible promoter P_groES, which showed an induction factor of 1300. When the genes htpG and pbpE, encoding a heat shock protein and a penicillin-binding protein, respectively, were fused to the promoter, the amount of recombinant product reached 10 and 13%, respectively, of the total protein [27].

### 2.2.2. Integrative Vectors

Integrative (or integration) vectors are one of the most powerful tools for editing the *Bacillus* genome. Differing from the autonomously replicating vectors, they do not replicate in *Bacillus* spp. cells (because they lack the origin of replication), but they can integrate into a specific place of the bacterial chromosome via homologous recombination and replicate as a part of it, thus avoiding vector segregation. Therefore, the greatest advantage of integrative vectors over autonomously replicating ones is the higher structural and segregational stability of the cloned fragments. The integration of a target sequence into a neutral site of the chromosome, as opposed to the normal locus, via double crossover is known as ectopic integration. The inserted DNA may be a plasmid, a PCR product, or even a fragment of genomic DNA from the same or different species, but in all cases, it should have 100 to 500 bp (or more) homology at the flanking regions with the target chromosome. Popular loci for ectopic integration are relatively inessential genes like amyE (for α-amylase) and lacA (encoding β-galactosidase), and they are frequently included in commercially available vectors [28]. The lacA locus integration was first applied in 2001 with the vector pAX01 [29].

The opportunities provided by integrative vectors are many and great. The most classic example of this is the knock-out mutant obtained by integration into and disruption of a target gene. A single crossover event (made possible by a single homologous sequence) is all that is necessary. Another famous application using the same mechanism is the fusion of the gene of interest with a reporter gene. This versatile technique is a relatively straightforward way to study gene expression quantitatively if the reporter is an enzyme (e.g., β-galactosidase, β-glucuronidase) or intracellularly if the reporter is easily visible (e.g., Green Fluorescent Protein). Various tags (FLAG, c-Myc, 6xHis, and 8xHis) may also be fused to the gene of interest, allowing its purification or immunoblot identification.
Ectopic integration via double crossover is now routinely performed with a great variety of target genes integrated into any region of the genome with a known sequence [30].

Rather than being synthesized de novo, integrative vectors are usually derived from other vectors. The classic shuttle vector pHY300PLK (Figure 1, Table 1), developed back in 1985 [31], has served as a template for the integrative vector pHYAMC, designed and successfully used for the disruption of amyE and prob (glutamate-5-kinase) in undomesticated B. subtilis strains [32]. Five integrative vectors for B. subtilis compatible with the BioBrick (RFC10) standard have also been developed. Three of them, pBS1C, pBS2E, and pBS4S, were derived from pDG1662, pAX01, and pDG1731 and designed to be integrated into the amyE, lacA, and thrC loci, respectively. The other two were reporter vectors, pBS1ClacZ with β-galactosidase (lacZ) and pBS3Clu with luciferase (luxABCDE), destined to be integrated into the amyE and sacA loci, respectively. All vectors contained RFC10-compatible multiple cloning sites. This mighty Bacillus “BioBrick Box” also provided five widely used tags that can be fused to the N- or C-terminus of target proteins: FLAG, His10, StrepII, HA, and c-Myc [33].

Potentially toxic proteins are difficult to handle, for obvious reasons, and require special systems for their recombinant production. One “gene expression toolkit” was designed for this purpose, an elaborate system of expression vectors (pMSE3, pBE-S, pH8201) and integration vectors (derivatives of pJET-lox-SSS and pJK, no fewer than 15 of them). Multiple deletions of all main extracellular proteases (nprB, mpr, aprE, nprE, vpr, epr, wprA, bpr), genes responsible for cell lysis (lytC) and sporulation (spoIIGA), and clusters for secondary metabolites (srfA, pksX) in B. subtilis JK32 yielded B. subtilis LS8P-D. The new strain was tested successfully with the production of two eukaryotic, traditionally difficult-to-express proteins: sulfhydryl oxidase (Sox) from Saccharomyces cerevisiae and human interleukin-1β [34].

Integrative vectors have been used on a vast scale in systems biology. Two deletion libraries, one kanamycin- and one erythromycin-resistant, were constructed in B. subtilis 168, in which every non-essential gene was substituted with an antibiotic-resistant cassette. Altogether, the libraries contain 3968 and 3970 genes for kanamycin and erythromycin, respectively. They open immense possibilities for the system-level understanding of Bacillus, and the authors were not slow to whet the appetite of the scientific community through some pilot studies. They refined the sets of essential and auxotrophic genes, identified several genes responsible for missing steps in the biosynthesis of serine, tyrosine, and phenylalanine, and determined the genes responsible for growth at low temperatures and the utilization of various carbon and nitrogen sources [35].

2.2.3. CRISPR/Cas9

The CRISPR/Cas9 system is the latest advance in genome editing. It allows for precision that is impossible to achieve with integrative vectors. The so-called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) were first discovered in the genome of E. coli in 1987, but after several decades of research, they seem to be present in about 50% of all bacteria. In combination with Cas proteins, which are dual RNA-guided endonucleases, CRISPR forms a complex but efficient system for combating invading DNA, a sort of microbial equivalent to adaptive immunity [36]. The system has been rapidly employed for genome editing in hosts ranging from E. coli and Streptococcus pneumoniae to Bombyx mori and Drosophila to plants, mice, and human cell lines [37].

Bacillus vectors with Cas9 and the requisite auxiliary sequences (e.g., sgRNA, single guide RNA) have been very popular in recent years. The vector pJOE899 was developed by Josef Altenbuchner in 2016 as a genome-editing tool for B. subtilis. It is a shuttle vector, with pUC ori for E. coli and temperature-sensitive pE194ts ori for B. subtilis, and two promoters, one mannone-inducible (Pmann) and one semisynthetic (Pvan*), the latter derived from the PvanABK promoter of Corynebacterium glutamicum and designed for high constitutive expression of sgRNA [38]. This versatile vector has since been modified, adapted, and used successfully in B. cereus [39] and B. megaterium [40]. Almost every vector can be
converted into a CRISPR/Cas9 vector with enough ingenuity. Even the old pHY300PLK (see Section 2.2.2) served as the basis for PHYcas9dsrf and five other Cas9-containing plasmids, all used for multiple knockouts in *B. subtilis* ATCC6051a. Although the efficiency was not high, between 33% and 53%, the final mutant B5, with five genes knocked out (*rfC*, *spoIIAC*, *nprE*, *aprE*, and *amyE*), demonstrated less foamy behavior during fermentation, greater resistance to spore formation, and 2.5-fold increased production of β-cyclodextrin glycosyltransferase [41].

One serious drawback of the system is its Cas9 toxicity to many wild-type *Bacillus* spp. This has necessitated the development of alternative systems. One of them includes vectors that lack the replication initiator protein gene (*rep*) and use the Rep proteins of another *Bacillus* strain that serves as a donor. The conjugal transfer between the donor and the recipient was achieved via the so-called Modified Integrative and Conjugative Element (MICE, see Section 2.3) and two integrative plasmids: pAD123, which contains the *rep* gene and may exist in both the donor and recipient; and the newly developed pSGC2IN, which contains DSO (Double-Strain Origin of Replication) but not *rep* and thus may exist in the recipient only after its chromosome has acquired the *rep* gene from pAD123 [42]. The system is laborious and error-prone, but it does provide an opportunity to work with Cas9-sensitive strains of *Bacillus* spp.

CRISPR interference (CRISPRi) is CRISPR/Cas9 employed for transcriptional inhibition of target genes. CRISPRi strains contain sgRNAs for Cas9 targeting the gene in question, resulting in the stalling of the RNA polymerase. The effect can be titrated, reducing expression to very low levels without eliminating it. The method is useful for studying essential genes or such that require conditional knockdown of transcription in order to examine a specific phenotype. It has found impressive applications in systems biology. A comprehensive essential gene-knockdown library of *B. subtilis* was constructed by this method in 2016 and used for drug target discovery. The authors tested their library with MAC-0170636, an antibiotic that upregulates the cell wall-damage-responsive promoter P*ywaC* by an unknown mechanism [43].

### Table 1. Selected vectors used in recombinant *Bacillus* spp.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Function</th>
<th>Size, bp</th>
<th>Selection 1</th>
<th>Features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMA5</td>
<td>Expression</td>
<td>7202</td>
<td>Kan, Amp</td>
<td>P&lt;sub&gt;hpa&lt;/sub&gt;, P&lt;sub&gt;AmpK&lt;/sub&gt;, f1 ori, repB</td>
<td>[21]</td>
</tr>
<tr>
<td>pBE-S</td>
<td>Expression</td>
<td>5938</td>
<td>Kan, Amp</td>
<td>P&lt;sub&gt;aprE&lt;/sub&gt;, SP&lt;sub&gt;aprE&lt;/sub&gt;, colE1 ori, pUB ori, His tag</td>
<td>[23]</td>
</tr>
<tr>
<td>pH4T43</td>
<td>Expression</td>
<td>8057</td>
<td>Amp, Cm</td>
<td>P&lt;sub&gt;grac&lt;/sub&gt;, SP&lt;sub&gt;amyQ&lt;/sub&gt;, LacI, ColE1</td>
<td>[44]</td>
</tr>
<tr>
<td>pHY300PLK</td>
<td>Expression</td>
<td>4870</td>
<td>Amp, Tet</td>
<td>ori-pAMa1, ori-177, repB</td>
<td>[31]</td>
</tr>
<tr>
<td>pHYAMC</td>
<td>Integration</td>
<td>7513</td>
<td>Amp, Tet</td>
<td>PApR, ori-pAMa1, ori-177, amyE'</td>
<td>[32]</td>
</tr>
<tr>
<td>pBacTag</td>
<td>Integration</td>
<td>5476</td>
<td>Amp, Ery</td>
<td>P&lt;sub&gt;grac&lt;/sub&gt;, lacI, ColE1 ori, tag 2</td>
<td>[45]</td>
</tr>
<tr>
<td>pHBintE</td>
<td>Integration</td>
<td>5683</td>
<td>Amp, Ery</td>
<td>P&lt;sub&gt;xylA&lt;/sub&gt;, repE, <em>E. coli</em> ori, <em>Bacillus</em> ori</td>
<td>[46]</td>
</tr>
<tr>
<td>pAX01</td>
<td>Integration</td>
<td>7781</td>
<td>Ery</td>
<td>P&lt;sub&gt;xylA&lt;/sub&gt;, xylR</td>
<td>[29]</td>
</tr>
<tr>
<td>pJOE8999</td>
<td>Editing</td>
<td>7794</td>
<td>Kan</td>
<td>Cas9, pUC ori, rep pE19ts, P&lt;sub&gt;manE&lt;/sub&gt;, P&lt;sub&gt;vanP&lt;/sub&gt;</td>
<td>[38]</td>
</tr>
<tr>
<td>PHYcas9dsrf</td>
<td>Editing</td>
<td>10,494</td>
<td>Amp, Tet</td>
<td>Cas9, P&lt;sub&gt;grac&lt;/sub&gt;, p15A ori, P&lt;sub&gt;amyQ&lt;/sub&gt;</td>
<td>[41]</td>
</tr>
</tbody>
</table>

1. Amp = Ampicillin; Kan = Kanamycin; Tet = Tetracycline; Ery = Erythromycin; Cm = Chloramphenicol. 
2. FLAG, c-Myc, 6xHis, 8His.

### 2.3. Methods for Vector Delivery

Natural transformation, a natural outcome of natural competence, is recognized as one of the major mechanisms of horizontal gene transfer and the single most important driving force of evolution in prokaryotes. At least one recent study argues that cell-to-cell natural transformation between different *B. subtilis* strains is a highly efficient process that remains underestimated. The authors report 66 transferrred DNA segments with an average
length of 27 kb [47]. Much more modest feats have been extremely difficult to achieve with other Bacillus strains.

The transformation of wild-type, undomesticated Bacillus is notoriously difficult. Electroporation has proved to be the most successful solution historically, but it took a great deal of time and effort to establish. A highly efficient protocol based on high-osmolarity media was developed and optimized for B. licheniformis and B. subtilis as late as 1999. The presence of relatively high concentrations of sorbitol and mannitol in the growth, electroporation, and recovery media was found to confer an almost 5000-fold increase in the transformation efficiency, reaching $1.4 \times 10^6$ transformants per µg DNA for Bacillus subtilis. Less impressive were the results with B. licheniformis: a 400-fold increase in the transformation efficiency and $1.8 \times 10^4$ transformants per µg of DNA. In both cases, a high field strength of 21 kV/cm was used [48]. The protocol has been successfully applied to B. telezensis 5RB [49]. Efficient electroporation methods have been described for B. thuringiensis [50], B. mycoides, a soil bacterium promoting plant growth [51], and B. subtilis ZK, a natural producer of iturin A [52].

Electroporation is not likely to disappear anytime soon, but recently developed recombinant methods certainly provide strong competition. Supercompetent strain B. subtilis SCK6 has been obtained through overexpression of the competence master regulator ComK. The comK gene from B. subtilis 168 was overexpressed in the SCK6 strain via the integrative vector pAX01 under the control of the strong xylose-inducible promoter $P_{\text{xylA}}$ [53]. Conjugal DNA transfer has recently emerged as a last-generation method for plasmid delivery, apparently preferable to both natural transformation (a similar but not identical process) and electroporation as far as undomesticated Bacillus are concerned. One recent study thoroughly explored the issue. The MICE system (Modified Integrative and Conjugative Element) was able to transform 41 undomesticated Bacillus subtilis strains and eight other Bacillus species (B. amyloliquefaciens, B. atrophaeus, B. lentus, B. licheniformis, B. megaterium, B. mojavensis, B. pumilus, and B. vallismortis). MICE was compared and found superior to natural competence, E. coli-to-Bacillus Conjugation (EBC) with E. coli S17-1 as a donor, and Bacillus-to-Bacillus Conjugation (BBC). MICE was the only system able to transform the domesticated B. subtilis 168 and 8 undomesticated B. subtilis strains. BBC was the second best, with 6 out of 8 undomesticated strains; EBC and electroporation managed only 4 out of 8; and natural competence was not even one [54].

3. Biotechnological Versatility of Bacillus spp.

An enormous variety of enzymes, growth factors, vitamins, peptides, amino acids, and low-MW compounds have been expressed in recombinant Bacillus spp. (Figure 2), often on a scale with industrial promise. Through the optimization of expression systems and developments in the field of bioengineering and the use of recombinant Bacillus strains, the highest values of industrially important target products have been achieved (Table 2).

3.1. Enzymes

Enzymes with applications in the food industry have predictably been in the spotlight. Genetic improvement of bacilli for the production of $\alpha$-amylase leads to a gradual increase in the yields obtained (Table 2). The goal of enhanced extracellular expression is achieved through signal peptide optimization and chaperone overexpression [55], the prevention of extracellular degradation by improving the folding environment [56], as well as by complex balancing of the entire secretion process [57]. Thus, the recombinant strain B. subtilis WHS9GSAB produced 35,779.5 U/mL $\alpha$-amylase for 93 h, reaching a productivity of 384.7 U/mL/h [58].
as by complex balancing of the entire secretion process [57]. Thus, the recombinant strain *B. subtilis* WHS9GSAB produced 35,779.5 U/mL α-amylase for 93 h, reaching a productivity of 384.7 U/mL/h [58].

**Figure 2.** Biotechnological versatility of *Bacillus* spp.

**Table 2.** Application of recombinant *Bacillus* spp. with the highest production of industrially important compounds.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vector</th>
<th>Compound</th>
<th>Genetic Source</th>
<th>Yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em> WHS11YSA</td>
<td>pHYYamySA</td>
<td>α-amylase</td>
<td><em>B. stearothermophilus</em></td>
<td>9201.1 U/mL</td>
<td>[55]</td>
</tr>
<tr>
<td><em>Brevibacillus choshinensis</em> (<em>B. brevis</em>)</td>
<td>pNCamyS-prsQ</td>
<td>α-amylase</td>
<td><em>B. stearothermophilus</em></td>
<td>17,925.6 U/mL</td>
<td>[56]</td>
</tr>
<tr>
<td><em>B. subtilis</em> WHS9GSAB</td>
<td>pHYGamySasecYEG</td>
<td>α-amylase</td>
<td><em>B. stearothermophilus</em></td>
<td>35,779.5 U/mL</td>
<td>[57]</td>
</tr>
<tr>
<td><em>Br. choshinensis</em> (<em>B. brevis</em>)</td>
<td>pNCMO2</td>
<td>β-amylase</td>
<td>*B. aryabhattai CCTCC M2017320</td>
<td>5371.8 U/mL</td>
<td>[58]</td>
</tr>
<tr>
<td><em>B. subtilis</em> WS9PUL</td>
<td>pHYcas9</td>
<td>pullulanase</td>
<td><em>B. deramificans</em></td>
<td>5951.8 U/mL</td>
<td>[59]</td>
</tr>
<tr>
<td><em>B. subtilis</em> WB600</td>
<td>pMA5</td>
<td>lipase A</td>
<td><em>B. subtilis</em></td>
<td>1164.9 U/mL</td>
<td>[60]</td>
</tr>
<tr>
<td><em>B. subtilis</em> DB10</td>
<td>pSKE194</td>
<td>xylanase</td>
<td><em>B. subtilis</em></td>
<td>1296 U/mg</td>
<td>[61]</td>
</tr>
<tr>
<td><em>B. licheniformis</em> MW3</td>
<td>pKVM1</td>
<td>2,3-butanediol</td>
<td><em>B. licheniformis</em></td>
<td>123.7 g/L</td>
<td>[62]</td>
</tr>
<tr>
<td><em>B. amyloliquefaciens</em> B 10-127</td>
<td>pMA5</td>
<td>2,3-butanediol</td>
<td><em>B. amyloliquefaciens</em></td>
<td>132.9 g/L</td>
<td>[63]</td>
</tr>
<tr>
<td><em>B. subtilis</em> 168</td>
<td>pMA5</td>
<td>acetoin</td>
<td><em>B. subtilis</em></td>
<td>91.8 g/L</td>
<td>[64]</td>
</tr>
<tr>
<td><em>B. subtilis</em> KH2</td>
<td>pKVM1 pMA5</td>
<td>poly-γ-glutamic acid</td>
<td><em>B. subtilis</em>, <em>B. licheniformis</em></td>
<td>23.28 g/L</td>
<td>[65]</td>
</tr>
<tr>
<td><em>B. subtilis</em> G600</td>
<td>T7-BOOST*</td>
<td>GABA †</td>
<td><em>B. subtilis</em></td>
<td>109.8 g/L</td>
<td>[66]</td>
</tr>
</tbody>
</table>

* T7-BOOST, T7-Based Optimized Output Strategy for Transcription, a system based on the inducible promoters P$_{hy-spank}$ and P$_{xylA}$; † GABA, γ-aminobutyric acid.
B. subtilis host strain 1A751 was engineered to express cellobiose 2-epimerase (CEase) from Thermoanaerobacterium saccharolyticum JW/YS485. CEase is a curious enzyme able to convert lactose to epilactose (4-O-β-d-galactopyranosyl-d-mannose), an epimer of lactose difficult to obtain with purely chemical synthesis. While lactose is one of the main by-products in the cheese industry, epilactose reportedly has prebiotic properties [67]. Food-grade maltogenic amylase (AmyM) was produced in the B. subtilis expression system based on the dal gene auxotrophic selection marker. The dal gene was deleted via the knockout plasmid pHYcas9dD, enabling selection with D-alanine instead of an antibiotic. The amylase activity reached 1364 U/mL on shake-flask cultivation and was scaled up to 2388 U/mL in a 3 L fermenter. The addition of maltogenic amylase in the process of breadmaking may extend the shelf life of bread [68].

Proteolytic enzymes used as detergents in the washing industry have received much attention. A metalloprotease from Planococcus sp. 11815 was expressed in Bacillus licheniformis 2709 and showed almost four times higher enzymatic activity (1186 vs. 291 U/mL) compared to the original bacteria [69]. Thermostable serine protease TTHA0724 from Thermus thermophilus HB8 was expressed in B. subtilis RIK1285 16.7 times more effectively than in E. coli. The enzyme showed promise as a detergent additive, being able to eliminate mites and completely clean protein stains at 60 °C. TTHA0724 may be of some use even in the food industry because of its ability to produce active soybean peptides with antioxidant properties at 75 °C [70].

The utilization of plant biomass, the most abundant and renewable energy source on the planet, rich in cellulose and other polysaccharides normally hard to degrade, has received a great deal of scientific attention. Although in this case Bacillus spp. are usually used as a genetic pool for the expression of target enzymes in E. coli, there are some interesting results with genetically modified Bacillus spp. as well. Recombinant B. subtilis DB104, harboring a pSKE194 plasmid with a gene for endoxylanase from B. subtilis AQ1, demonstrated a higher ability to degrade xylan than the non-recombinant strain. The xylanase activity was further increased twice in a 4.5 L fermenter with an inexpensive medium from agricultural waste, finally reaching 602 U/mL after 48 h. Xylan, the second most abundant plant polysaccharide after cellulose, is the source of valuable derivatives such as xylooligosaccharides (XOS), potentially useful as prebiotics in functional foods [61].

3.2. Growth Factors, Vitamins, and Amino Acids

Growth factors have a long and rich expression history in Bacillus. One recent study achieved 104 mg/L human Epidermal Growth Factor (hEGF) within 24 h in B. subtilis DB-104 under the control of an optimized promoter, P_{sdp}-4 [74]. This is a spectacular amount compared to the now historical value of 7 mg/L hEGF obtained a quarter of a century ago [75], not to mention the less than 1.2 g/L previously achieved by B. brevis HDP31,
which, moreover, took 6 days to accumulate [76]. However, it must be noted that the quantification of Jun et al. [69] was conducted with software processing of protein bands on SDS-PAGE and should be confirmed with a more reliable quantitative method. The insulin-like growth factor 1 (IGF-1), a small peptide of 70 amino acids vital for gastrointestinal health, and the basic fibroblast growth factor (bFGF), a key player in wound healing, have also been expressed in recombinant Bacillus. B. subtilis WB800N, pHT43 vector, and the novel fusion tag DAMP4 were used to obtain 17 mg/L IGF-1/DAMP4 fusion protein, but only 2.5 mg/L tag-free recombinant IGF-1—a tiny but not unusual amount for that growth factor [77]. B. subtilis 1A751 was engineered to produce bFGF fused to the cellulose-binding domain (CellBD) of the endoglucanase gene cenA from Cellulomonas fimi and ssp DnaB, one of the so-called “inteins” (also known as “protein introns”) found in some bacteria and widely employed for expression and purification of recombinant proteins. The result was the auto-processing of the CellBD-DnaB-bFGF fusion construct and the relatively high yield of 84 mg/L of biologically active bFGF [78].

Vitamins have also been successfully expressed in Bacillus, but not necessarily in industrial amounts. Cobalamin (Vitamin B₁₂) production by B. megaterium ATCC10778 was achieved as far back as 1986, though in rather small amounts: about 26 µg/L [79]. Much more recently, B. megaterium DSM509 was subjected to overexpression of the genes responsible for cobalamin biosynthesis, particularly the operons hemAXCDBL (6.2 kb) and cobl (10.5 kb), under a strong promoter induced by xylose (PₓylA) and via chromosomal integration with the vector pHBintE. Despite the 8- to 20-fold increase in the intracellular concentration of cobalamin in transformants compared to the wild type, the final vitamin concentrations remained very low, 1–1.5 µg/L [80]. Riboflavin (Vitamin B₂) has been obtained from B. subtilis RX10 via overexpression of ykgB, encoding 6-phosphogluconate-1,5-lactonase, the enzyme catalyzing the second step of the Pentose Phosphate Pathway. This metabolic readjustment assured increased levels of ribose-5-phosphate, a major substrate, and ultimately 7 g/L riboflavin [81]. An even more impressive result was achieved with B. subtilis RF1, in which, among other things, the vgb gene encodes hemoglobin from Vitreoscilla sp. As a result of the increased oxygen utilization, the production of riboflavin was 45.51% higher than the parent strain and reached 10.71 g/L in a 5 L bioreactor [82]. Riboflavin production by Bacillus has been the subject of other remarkable feats of metabolic engineering, as discussed in more detail later (Section 5.2).

Some work has been carried out on amino acids, including some of their valuable derivatives. The promising drug for treating Parkinson’s disease, l-DOPA (3-Hydroxy-l-tyrosine), was produced in substantial amounts by an engineered strain, Bacillus licheniformis, in which, among other things, a tyrosine hydroxylase from Streptosporangium roseum DSM 43021 was introduced. The highest yield reached was 167 mg/L in shake flasks (2.41 times higher than the parent strain) and 1290 mg/L in a 15 L bioreactor [83].

3.3. Antimicrobial and Immunization Peptides

Antimicrobial peptides (AMPs) have received much attention in recent years as an alternative to antibiotics. Abaecin, an antimicrobial peptide from Apis mellifera that acts as an enhancer of the pore-forming effect of antimicrobial peptides, was expressed and purified from the supernatant of B. subtilis. The recombinant abaecin did not inhibit the growth of E. coli K88, but it did enhance the effect of sublethal doses of cecropin A and hynemoptaeacin, both bactericidal proteins isolated from the venom of Apis mellifera [84]. Porcine β-defensin-2 (pBD-2) and cecropin P1 (CP1) were expressed as a fusion antimicrobial peptide in B. subtilis 168 via the pMK4 vector. pBD2-CP1 was digested by enterokinase, and the separate peptides were tested against various pathogens (E. coli, Salmonella typhimurium, Haemophilus parasuis, and Staphylococcus aureus), revealing potent antimicrobial activities. The recombinant B. subtilis strain was shown to promote the health of piglets when added to their feed [85]. A novel antimicrobial peptide derived from the large yellow croaker (Larimichthys crocea) was identified via the expression system of B. subtilis SCK6. The new peptide was given the name Lc1687, was found to consist of 51 amino acids, and
showed strong activity against various Gram (−) and Gram (+) pathogens, including *St. aureus* and *Vibrio vulnificus* [86].

Recombinant strains of *B. subtilis* have entered even the field of immunology as vital components of vaccines. *B. subtilis*, capable of secreting the capsid protein (Cap) of porcine circovirus type 2 (PCV2), one of the most serious pathogens in pigs worldwide, was found to improve the immune response in mice. The authors used the capsid protein from PCV2d, the type currently prevalent in Chinese pigs, and observed it in the supernatant of recombinant bacteria virus-like particles (VLP) of PCV2d Cap protein [87]. Oral immunization of chickens with *B. subtilis* expressing the multi-epitope protein OmpC-FliC-SopF-SseB-IL-18 was shown to stimulate their immune response towards *Salmonella* Enteritidis, a major threat for poultry and the cause of massive economic losses [88].

### 3.4. Low-MW Compounds

Platform chemicals like 2,3-butanediol (2,3-BD) and acetoin, previously derived from oil, can now be obtained in a more environment-friendly way. A number of microorganisms, including many *Bacillus* spp., have demonstrated strong producing abilities, in a few exceptional cases reaching about 15%, after the proper genetic modification [89].

In the case of 2,3-BD, microbial production can be tailored to specific stereoisomers; three of them exist for 2,3-BD, a meso compound, and two enantiomers, D(−) and L(+). Meso-2,3-butanediol production has been substantially achieved with *B. licheniformis* WX-02, 98 g/L [90], and with *B. subtilis* BSF9, 103.7 g/L [91]. The latter study abolished the production of D(−)-2,3-BD by deleting the gene for the respective butanediol dehydrogenase (BDH). Two stereospecific BDHs were also identified in *B. licheniformis* MW3. Their deletion yielded two different strains capable of producing 90.1 g/L of the meso compound after 32 h of fermentation and 123.7 g/L of the D(−) enantiomer after 42 h of fermentation [62]. The most spectacular 2,3-BD titers so far have been achieved with *B. amyloliquefaciens* B10-127, 102.3 g/L [92] and 132.9 g/L [63], both times by sophisticated manipulation of the NADH/NAD⁺ system and selective knock-out of relevant genes. In these cases, however, the exact isomer is not specified.

From an industrial point of view, the price of the substrate is no less important than the final amount of the product. Broadening the substrate spectrum into cheaper regions is a perennial challenge in industrial microbiology, including 2,3-BD production. *B. licheniformis* 24, a native superproducer of 2,3-BD, was designed to utilize inulin, a cheap and renewable polysaccharide from plant biomass; for this purpose, an inulinase (fructan-β-fructosidase) from *Lacticaseibacillus paracasei* DSM 23505 was cloned into the pBE-S shuttle vector and heterologously expressed. While the overall production of 2,3-BD remained low (18.5 g/L after 7 days of fermentation with 200 g/L chicory flour), the recombinant *B. licheniformis* 24 showed more than 50% higher titers of 2,3-BD and acetoin combined than the wild type after 6 days of fermentation. Moreover, while the wild type’s 2,3-BD titer declined sharply in the next three days, that of the recombinant was correspondingly increased. This curious effect produced a 7-fold gap between the strains after 9 days of fermentation [93]. Acetoin, which is not only a platform chemical but a popular flavor, has been synthesized by various recombinant strains of *B. subtilis*, such as BS-ppb11, which achieved 82.2 g/L [94]. A metabolically engineered version of *B. subtilis* 168 reached 91.8 g/L acetoin and 2.3 g/L/h productivity, mostly due to a decreased NADH/NAD⁺ ratio (2.2-fold) [64]. Somewhat lower but still impressive titers have been obtained with *B. licheniformis* strains: 64 g/L with MW3 [95] and 79 g/L with WX0279 [96]. The main genetic modification in both cases was the deletion of *budC* (2,3-butanediol dehydrogenase) and *gdh* (glycerol dehydrogenase).

### 4. Genetic Engineering in *Bacillus* spp.

Genetic engineering has almost infinite possibilities in *Bacillus* spp. It has become possible to engineer and fine-tune these microbial factories to a degree unthinkable a few decades ago. A wide range of integrative vectors have been used to influence gene expression on virtually every level, from transcription and mRNA stability to extracellular export.
Knockout and overexpression of multiple genes have made possible the manipulation of whole metabolic pathways [97]. The latest developments in Bacillus bioengineering are summarized in Figure 3 and discussed further in this section.

![Figure 3. Genetic engineering in Bacillus spp.](image)

4.1. Heterologous Expression with Limited Modification

4.1.1. Constitutive Promoters

Classic and relatively strong constitutive promoters such as P_{434}, P_{veg}, and P_{lpall} have been employed in the heterologous expression of a wide variety of target proteins, from enzymes and amino acids all the way to synthetic peptides and growth factors, with minimum genetic modification (Table 3). Trypsin from *Streptomyces populi* A249 [98] and L-asparaginase from *B. cereus* BDRD-ST26 [99] were successfully expressed in *B. subtilis* via simple cloning into shuttle vectors. In the case of L-asparaginase, an enzyme valued for its ability to reduce acrylamide levels in foods, 20-fold higher activity was observed when it was introduced from *B. cereus* BDRD-ST26 into *B. subtilis* WB600. More importantly, this higher activity is coupled with a 72% reduction in acrylamide levels in pretreated potato strips. Other Bacillus spp., such as *B. licheniformis* and *B. velezensis*, have also been used for the purpose of, for example, the expression of cellulase genes from the highly efficient cellulosome of *Acetivibrio thermocellus* with the prospect of utilizing lignocellulosic biomass [49]. Signal peptides (SP) can be added to the vector, translated into the final protein, and influence its secretory fate. The enzyme β-agarase from *Pseudomonas hodoensis* was overexpressed in *B. subtilis* RIK1285, and its extracellular secretion improved by 44% when SP_{agrE} was exchanged with SP_{lipA} [100]. Human epidermal growth factor (hEGF), crucial for wound healing, was secreted almost twice as efficiently with SP_{synD} (the signal peptide of endo-1,4-beta xylanase) than with SP_{lipA} [101]. Signal peptides can be substituted with any other useful sequence. The production of L-theanine, a glutamate analog used for improving brain function even though there is no valid scientific evidence that it does, was achieved by introducing a novel γ-glutamyltranspeptidase (GGT, encoded by ggt) from *B. pumilus* ML413 into *B. subtilis* 168. The addition of a poly(A/T) tail to the 3′-end of ggt increased the mRNA stability by 58% and GGT activity by 60% [102].

It must be kept in mind, however, that even the simplest genetic modifications are not an end in themselves. Various other factors need to be considered, not least the nature of the host. To give but one example of the superiority of *Bacillus* over *E. coli*, food-grade sucrose phosphorylase, an enzyme employed in the production of kojibiose (a disaccharide and prebiotic, inhibitor of α-glucosidase), was transplanted from *Bifidobacterium adolescentis* to *B. subtilis* (CCTCC M 2016536). Secretion was achieved even without a signal peptide, and the extracellular enzyme activity proved to be 3.5-fold higher than it was possible to obtain with the same experimental design in *E. coli* [103].

Even the specific strain may be of great importance. Pullulanase, a debranching enzyme with industrial application in starch processing, from *B. naganoensis* JNB-1 was
expressed in *B. subtilis* WB800 and WB600. A simple promoter change from \( P_{hpaII} \) to \( P_{43} \) was sufficient to increase the pullulanase activity more than six times: from 3.9 U/mL in WB800 to 24.5 U/mL in WB600. It is fascinating to note that nearly half of that effect is due not to the promoter but to the host strain. When pMA9011 and \( P_{43} \) were used in WB800, the activity reached only 8.7 U/mL, nearly 3 times (2.82 to be precise) lower than the same vector and the same promoter in WB600 [104].

4.1.2. Inducible Promoters

Inducers come in a splendid variety. They may assume the form of specific compounds like IPTG, various substrates such as sugars (maltose, sucrose, glucose), or even different types of environmental stress (temperature, pH, salts). Inducible promoters thus offer a range of opportunities unthinkable to their constitutive colleagues. Sometimes, a simple promoter exchange is enough to produce a remarkable effect. Creatinase, an enzyme degrading creatinine and thus of vital importance for studying renal function, was produced almost five times more by *B. subtilis* 1A751 when the constitutive \( P_{hpaII} \) from the PMA5 vector was exchanged with the inducible \( P_{glv} \) [105].

The combination of the same maltose-inducible promoter \( P_{glv} \) and modest genetic modification has been used successfully for the production of various synthetic peptides. These include T9W, a variant of the pig myeloid antimicrobial peptide-36 (MAP 36) that displays efficient and specific activity against *Pseudomonas aeruginosa* [106]; PR-FO, a novel \( \alpha \)-helical hybrid antimicrobial peptide with strong activity and high stability [107]; and cecropin AD (CAD), a hybrid peptide of 37 amino acids with strong antibacterial and antitumor properties and no hemolytic activity, which is regarded as a promising antibiotic candidate [108].

IPTG (isopropyl-\( \beta \)-D-thiogalactoside) is the most popular inducer, especially when coupled with the \( P_{grac} \) and \( P_{spac} \) promoters. However, contradictory reports about the cost and toxicity of IPTG [109,110] cast some doubt on its industrial and food-grade applications, respectively. Nevertheless, IPTG remains widely used, and IPTG-inducible promoters like \( P_{spac} \) and \( P_{grac} \) (also known as \( P_{grac01} \)), strong enough in the first place, have been further developed into even more robust versions. \( P_{grac01} \) is 50 times stronger than \( P_{spac} \) and has been used for the production of human bone morphogenetic protein-2 (hBMP2), a molecule with important applications in spine fusion and ortho/maxillofacial surgeries [111]. \( P_{grac212} \) differs from \( P_{grac01} \) by the addition of the mRNA-controllable stabilizing element (CoSE) and has shown great promise with the Human Rhinovirus 3C Protease (HRV3C) as a reporter [112].

Naturally, inducible promoters can also be combined with an almost infinite variety of signal peptides, tags, tails, and the like. SUMO (small ubiquitin-related modifier), a fusion tag of approximately 100 amino acids acting as a secretory enhancer and folding catalyst, has been especially popular in recent years, usually in combination with tags for affinity purification such as 6xHis [98] and StrepII [113]. The latter study also employed a signal peptide (SP\(_{YoaW}\)) to achieve 5–6 times higher extracellular activity with alkaline phosphatase (PhoA) as a reporter gene. Vectors may also carry various non-fusion proteins. Three enhancers (DegQ, DegU, and DegS) were cloned in pMA0911 and studied for their effect on the activity of pullulanase from *B. naganoensis* introduced into *B. subtilis* WB800. The strongest impact, increasing pullulanase activity by 60%, was obtained with DegQ, a small peptide of 46 amino acids known to stimulate the expression of many degradation enzymes. The pullulanase activity was further enhanced by placing degQ closer to the sucrose-inducible promoter \( P_{sacB} \)—26.5 U/mL; an almost 6-fold increase compared to the original strain without any enhancer [114].
Table 3. Heterologous expression in *Bacillus* spp. with limited modification.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Signal Peptide</th>
<th>Vector</th>
<th>Genetic Modifications ¹</th>
<th>Target Compound ²</th>
<th>Source ³</th>
<th>Host ³</th>
<th>Effect ⁴</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₄₃</td>
<td>SP&lt;sub&gt;amyE&lt;/sub&gt;</td>
<td>pP43NMK</td>
<td>cloning of ASN (<em>BcA</em>)</td>
<td>L-asparaginase</td>
<td><em>B. cereus</em></td>
<td><em>Bs WB600</em></td>
<td>20-fold higher BcA activity; 72% decrease of acrylamide in pretreated potato strips</td>
<td>[99]</td>
</tr>
<tr>
<td>P₄₃</td>
<td>SP&lt;sub&gt;sacB&lt;/sub&gt;</td>
<td>pWB980</td>
<td>cloning of GM2938</td>
<td>trypsin</td>
<td><em>Streptomyces populi</em></td>
<td><em>Bs SCK6</em></td>
<td>1622 U/mL esterase activity and 34 U/mL amidase activity for purified GM2938</td>
<td>[98]</td>
</tr>
<tr>
<td>P&lt;sub&gt;aprE&lt;/sub&gt;</td>
<td>-</td>
<td>pBE-S</td>
<td>cloning of cel8A and cel48S</td>
<td>2 cellulases</td>
<td><em>Acetivibrio thermocellus</em></td>
<td><em>Bl 24</em></td>
<td>7-fold higher EA for Cel8a in <em>Bl</em> 24 and Cel48S in <em>Bv</em> 5RB</td>
<td>[49]</td>
</tr>
<tr>
<td>P&lt;sub&gt;aprE&lt;/sub&gt;</td>
<td>SP&lt;sub&gt;lipA&lt;/sub&gt;</td>
<td>pBE-S</td>
<td>SP exchange</td>
<td>β-agarase</td>
<td><em>Ps. hodoensis</em></td>
<td><em>Bs RIK1285</em></td>
<td>44% higher secretion than SP&lt;sub&gt;aprE&lt;/sub&gt;</td>
<td>[100]</td>
</tr>
<tr>
<td>P&lt;sub&gt;hpaiI&lt;/sub&gt;</td>
<td>-</td>
<td>pBSMuL3</td>
<td>host exchange</td>
<td>sucrose phosphorylase</td>
<td><em>Bifidobacterium adolescentis</em></td>
<td><em>Bs CCTCC M2016536</em></td>
<td>3.5-fold higher extracellular EA than cloning in <em>E. coli</em></td>
<td>[103]</td>
</tr>
<tr>
<td>P₄₃</td>
<td>-</td>
<td>pMA0911</td>
<td>P&lt;sub&gt;hpaiI&lt;/sub&gt; exchanged for P₄₃</td>
<td>pullulanase</td>
<td><em>B. naganoensis</em> JNB-1</td>
<td><em>Bs WB600</em></td>
<td>6-fold higher EA than the same vector with P&lt;sub&gt;hpaiI&lt;/sub&gt; in <em>Bs WB800</em></td>
<td>[104]</td>
</tr>
<tr>
<td>P&lt;sub&gt;hpaiI&lt;/sub&gt;</td>
<td>-</td>
<td>pMA5</td>
<td>poly(A/T) tail added to 3'-end of ggt</td>
<td>L-theanine</td>
<td><em>B. pumilus</em> ML413</td>
<td><em>Bs 168</em></td>
<td>Poly(A/T) increased mRNA stability by 58% and GGT activity by 60%; 53 g/L after 16 h</td>
<td>[105]</td>
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<tr>
<td>P&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>-</td>
<td>pJOE-8739</td>
<td>deletion of sporulation genes; promoter change</td>
<td>γ-PGA</td>
<td><em>Bs 168</em></td>
<td><em>Bs IIG-Bs2</em></td>
<td>129% higher carbon yield with glucose as a source</td>
<td>[115]</td>
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<tr>
<td>T7</td>
<td>SP&lt;sub&gt;xynD&lt;/sub&gt; (lypo type)</td>
<td>pDMT pDBT</td>
<td>2 copies of hEGF cassette; ΔmprB; Δmpr</td>
<td>human epidermal growth factor (hEGF)</td>
<td><em>Homo sapiens</em></td>
<td><em>Bs PT5, PT6, PT7</em></td>
<td>Almost a 2-fold increase due to SP; 12% more with 2 copies of hEGF</td>
<td>[102]</td>
</tr>
<tr>
<td>P&lt;sub&gt;sacB&lt;/sub&gt;</td>
<td>SP&lt;sub&gt;lipA&lt;/sub&gt;</td>
<td>pMA0911</td>
<td>enhancers DegQ, Deg5, DegU</td>
<td>pullulanase</td>
<td><em>B. naganoensis</em></td>
<td><em>Bs WB800</em></td>
<td>5.9-fold higher activity with DegQ</td>
<td>[114]</td>
</tr>
<tr>
<td>P&lt;sub&gt;glv&lt;/sub&gt;</td>
<td>SP&lt;sub&gt;lipA&lt;/sub&gt;</td>
<td>pMA5</td>
<td>P&lt;sub&gt;hpaiI&lt;/sub&gt; discarded</td>
<td>creatinase</td>
<td>-</td>
<td><em>Bs 1A751</em></td>
<td>5-fold higher EA than P&lt;sub&gt;hpaiI&lt;/sub&gt;</td>
<td>[105]</td>
</tr>
<tr>
<td>P&lt;sub&gt;glv&lt;/sub&gt;</td>
<td>-</td>
<td>pGJ148</td>
<td>6xHis-SUMO tag</td>
<td>T9W</td>
<td>synthetic</td>
<td><em>Bs WB800N</em></td>
<td>2.3 mg/L purified T9W</td>
<td>[106]</td>
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Table 3. Cont.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Signal Peptide</th>
<th>Vector</th>
<th>Genetic Modifications</th>
<th>Target Compound</th>
<th>Source</th>
<th>Host</th>
<th>Effect</th>
<th>Reference</th>
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<td>P&lt;sub&gt;glv&lt;/sub&gt;</td>
<td>SP&lt;sub&gt;sacB&lt;/sub&gt;</td>
<td>pGJ148</td>
<td>-</td>
<td>cecropin AD (CAD)</td>
<td>synthetic</td>
<td>Bs WB800N</td>
<td>24.6 mg/L CAD, 93% purity, similar antimicrobial activity to synthetic CAD</td>
<td>[107]</td>
</tr>
<tr>
<td>P&lt;sub&gt;glv&lt;/sub&gt;</td>
<td>SP&lt;sub&gt;sacB&lt;/sub&gt;</td>
<td>pGJ148</td>
<td>-</td>
<td>PR-FO</td>
<td>synthetic</td>
<td>Bs WB800N</td>
<td>3–4 mg/L purified PR-FO</td>
<td>[99]</td>
</tr>
<tr>
<td>P&lt;sub&gt;grac&lt;/sub&gt;</td>
<td>SP&lt;sub&gt;graW&lt;/sub&gt;</td>
<td>pIJH</td>
<td>SP&lt;sub&gt;graW&lt;/sub&gt; fused with StrepII-SUMO</td>
<td>alkaline phosphatase (r)</td>
<td>-</td>
<td>Bs WB800N Bs KO7A</td>
<td>5–6 times higher activity than with SP&lt;sub&gt;amyQ&lt;/sub&gt;</td>
<td>[107]</td>
</tr>
<tr>
<td>P&lt;sub&gt;grac01&lt;/sub&gt;</td>
<td>-</td>
<td>pHt43 pTz57R/BMP2</td>
<td>-</td>
<td>human bone morphogenetic protein-2 (rhBMP2)</td>
<td>Homo sapiens</td>
<td>Bs SCK6 Bs WB600</td>
<td>5–9 mg/L</td>
<td>[111]</td>
</tr>
<tr>
<td>P&lt;sub&gt;grac212&lt;/sub&gt;</td>
<td>-</td>
<td>pHT212</td>
<td>solubility tag at the N-terminus</td>
<td>HRV3C (r)</td>
<td>Homo sapiens</td>
<td>Bs 1012</td>
<td>8065 U/mg for purified protease</td>
<td>[112]</td>
</tr>
</tbody>
</table>

1 SP = Signal peptide; ggt = γ-glutamyltranspeptidase; 2 (r) = reporter; γ-PGA = poly-γ-glutamic acid; HRV3C = Human Rhinovirus 3C Protease; 3 B. = Bacillus; Bs = Bacillus subtilis; Bl = Bacillus licheniformis; Bv = Bacillus velezensis; Ps = Pseudomonas; 4 EA = Enzyme activity.
4.2. Promoter Engineering in Bacillus spp.

4.2.1. Self-Inducible Systems

Promoters that require no specific compound for their activation are known as self-inducing or auto-inducing. The role of the inducer is usually assumed by the growth phase or some type of environmental stress (temperature, pH). \( P_{srfA} \), the efficient cell-density-dependent auto-inducible promoter of the \( srf \) operon (four genes for surfactin synthetase), has shown some promise in this direction. When the core sequences from \((-10)\) to \((-35)\) were substituted with the consensus motifs TATAAT and TTGCAT, respectively, 1.7-fold higher overexpression of aminopeptidase was obtained compared to the relatively strong promoter \( P_{hpall} \) [116]. \( P_{23} \), a dual promoter (\( P_{srfA} - P_{hpall} \)) obtained from a library of \( P_{srfA} \) derivatives, showed inducer-free activity related to cell density and 2.5-fold higher promoter activity than \( P_{srfA} \) with GFP as a reporter [117]. \( P_{srfA} \) is also useful in the food-grade production of different molecules, for example, the beef meaty peptide (BMP), an umami-flavored peptide with a bright future in food biotechnology [118].

Inducer-free vectors have been obtained from their IPTG-inducible colleagues by deleting parts of the \( lacI \) gene and encoding the LacI repressor, which determines the induction. The promoters \( P_{grac01} \) and \( P_{grac100} \) (Table 4) were tested under these circumstances and showed inducer-free expression levels of \( \beta \)-galactosidase comparable to those with inducers [119]. Inducer-free integrative vectors have also produced high expression levels under the control of \( P_{grac212} \). Integration into the \( amyE \) and \( lacA \) loci of the \( B. subtilis \) chromosome yielded 53.4% higher expression [120]. Another remarkable self-inducing promoter is \( P_{gibz} \) chosen as the most potent (more than five times higher activity than \( P_{43} \) on \( \beta \)-galactosidase assay) from 11 promoters selected via \( B. subtilis \) microarray data and qPCR, and able to drive high expression of target proteins during the stationary phase without inducer, as shown by the 2.3- and 7.4-fold higher expression of organophosphorus hydrolase and pullulanase, respectively, compared to \( P_{43} \) [121].

Autoinducible expression systems from other bacteria may also be used. The LuxRI quorum sensing system of \( Aliivibrio fischeri \) was successfully introduced into \( B. subtilis \) K07. The two-component system was devised, consisting of an induction module (\( S \)) with \( luxR \) and \( luxI \) from \( A. fischeri \) (under their respective promoters, \( P_{luxR} \) and \( P_{luxI} \)) and a response module (\( R \)) with the luminescence operon \( luxABCDE \) from the pBS3Clux plasmid. The regions \((-40)\) and \((-10)\) were further optimized by introducing enhancing mutations. The S1-R6 construct showed a 2.5 to 3.2 times stronger promoter response than \( P_{srfA} \) and \( P_{veg} \), respectively. This is a remarkable achievement because \( P_{veg} \) is considered one of the strongest constitutive promoters in \( B. subtilis \) [122].

4.2.2. Promoter Remodeling

Promoters have proved to be a versatile platform for genetic manipulation. Several studies have explored the possibilities of promoter remodeling.

Much has been made of \( P_{srfA} \), though with moderate success so far. A promoter library was constructed by randomized mutation of the \((-10)\) region, but the best member of it, \( P_{V1} \), showed only about 1.6-fold higher expression levels with GFP and aspartase as reporters [123]. Five synthetic promoters were obtained with mutations in the \((-35)\) and \((-10)\) regions of \( P_{srfA} \), but the most potent of them, \( P_{04} \), conferred only a 30% increase in the production of recombinant nattokinase, a fibrinolytic serine protease originally derived from the Japanese food natto, with promising applications in the prevention of cardiovascular diseases. Approximately the same benefit was conferred by the exchange of \( SP_{epr} \) for \( SP_{wapA} \), a neat reminder of the importance of signal peptides [124].
### Table 4. Promoter engineering in *Bacillus* spp.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Signal Peptide</th>
<th>Vector</th>
<th>Genetic Modifications</th>
<th>Target</th>
<th>Source</th>
<th>Host</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{srfA}$</td>
<td>SP AP</td>
<td>pMA09</td>
<td>8BMP (multi-copy BMP) autoinduced</td>
<td>BMP</td>
<td>-</td>
<td>Bs 168</td>
<td>successful expression and purification with industrial promise</td>
<td>[118]</td>
</tr>
<tr>
<td>mut$P_{srfA}$</td>
<td>SP AP</td>
<td>pBSG01 pMA05</td>
<td>(-10) and (-35) core sequences substituted with consensus sequences</td>
<td>aminopeptidase (AP)</td>
<td>Bs Zj016</td>
<td>Bs 168</td>
<td>1.7-fold AP overexpression compared to the $P_{hypA}$ promoter; confirmed on protein level</td>
<td>[116]</td>
</tr>
<tr>
<td>$P_{23}$</td>
<td>-</td>
<td>pAX-01 pBSG03</td>
<td>library of $P_{srfA}$ derivatives; chromosome integration; 12 dual promoters tested</td>
<td>GFP</td>
<td>Bs Zj016 Bs natto</td>
<td>Bs BSG1682</td>
<td>2.5-fold stronger promoter activity than $P_{srfA}$</td>
<td>[117]</td>
</tr>
<tr>
<td>$P_{grac01}$</td>
<td>-</td>
<td>pHT1655</td>
<td>lacI removal</td>
<td>$\beta$-galactosidase (r) inducer-free</td>
<td>-</td>
<td>Bs 1012</td>
<td>Expression levels are similar to those with induction</td>
<td>[119]</td>
</tr>
<tr>
<td>$P_{grac100}$</td>
<td>-</td>
<td>pHT2080</td>
<td>genome integration at $lacA$ or $amyE$ locus</td>
<td>$\beta$-galactosidase (r) inducer-free</td>
<td>-</td>
<td>Bs 1012</td>
<td>53.4% higher expression after integration into the chromosome</td>
<td>[120]</td>
</tr>
<tr>
<td>$P_{luxR}$</td>
<td>-</td>
<td>pBS3Clux</td>
<td>expression system based on $luxR$ and $luxI$; (-40) and (-10) regions optimized</td>
<td>riboflavin</td>
<td>$Alilvibrio fischeri$ Bs 168</td>
<td>Bs K07</td>
<td>2.5 to 3.2 times stronger promoter responses than $P_{srfA}$ and $P_{veg}$</td>
<td>[122]</td>
</tr>
<tr>
<td>$P_{phg}$</td>
<td>SP $amy$</td>
<td>pUBC19</td>
<td>11 promoters tested: $\alpha$-amylase SP from <em>B. amyloliquefaciens</em> pululanase organophosphorus hydrolase</td>
<td>pullulanase $B. naganonensis$ $Pc$ $P. pseudoalcaligenes$</td>
<td>Bs WB600</td>
<td>Bs WB600</td>
<td>7.4 times higher activity than $P_{43}$ 2.3 times higher activity than $P_{43}$ no inducer in both cases</td>
<td>[121]</td>
</tr>
<tr>
<td>Pn</td>
<td>-</td>
<td>pBSG03</td>
<td>randomized mutations adjacent to the (-10) region</td>
<td>aspartase (r)</td>
<td>-</td>
<td>Bs 168</td>
<td>1.6-fold higher transcriptional activity than $P_{srfA}$ after 12 h</td>
<td>[123]</td>
</tr>
<tr>
<td>P04</td>
<td>SP $wapA$</td>
<td>pMA0911</td>
<td>mutations in -35 and -10 regions of $P_{srfA}$; Cis-acting CodY at 5'-UTR</td>
<td>nattokinase</td>
<td>-</td>
<td>Bs WB600 Bs WB800</td>
<td>~30% higher EA with SP $wapA$ than SP $epr$; further ~30% increase with best of 5 synthetic promoters</td>
<td>[124]</td>
</tr>
<tr>
<td>$P_{BH4}$</td>
<td>-</td>
<td>pAX01 pBSG03</td>
<td>synthetic promoter library</td>
<td>$\beta$-glucuronidase nattokinase</td>
<td>-</td>
<td>Bs WB600</td>
<td>3 times greater promoter strength than $P_{srfA}$</td>
<td>[125]</td>
</tr>
</tbody>
</table>
Table 4. Cont.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Signal Peptide</th>
<th>Vector</th>
<th>Genetic Modifications</th>
<th>Target</th>
<th>Source</th>
<th>Host</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{groc100}$</td>
<td>-</td>
<td>pHT100</td>
<td>UP of $P_{groc0}$ element optimized</td>
<td>$\beta$-galactosidase (r)</td>
<td>-</td>
<td>Bs 1012</td>
<td>9.2 times higher expression compared to $P_{groc0}$</td>
<td>[126]</td>
</tr>
<tr>
<td>P43′–riboE1</td>
<td>-</td>
<td>pBSG03</td>
<td>$P_{43}$ combined with theophylline riboswitch; 9-bp spacer SD; and start codon</td>
<td>$\beta$-glucuronidase (r)</td>
<td>-</td>
<td>Bs 168</td>
<td>switch from constitutive to inducible expression</td>
<td>[127]</td>
</tr>
<tr>
<td>$P_{groE}$</td>
<td>SPamyQ</td>
<td>pHT43</td>
<td>lac operator from <em>E. coli</em> added</td>
<td>nanobodies</td>
<td>Camelida</td>
<td>Bs WB800N</td>
<td>successful IPTG-induced production</td>
<td>[128]</td>
</tr>
<tr>
<td>$P_{hpaII}$–P$_{amyQ}$</td>
<td>-</td>
<td>pP43NMK</td>
<td>RBS site modification</td>
<td>pullulanase</td>
<td>-</td>
<td>Bs 168</td>
<td>136.8 times higher activity than the wild type</td>
<td>[129]</td>
</tr>
<tr>
<td>$P_{amyE}$–cdd</td>
<td>SPac</td>
<td>pP43NMK</td>
<td>33 promoters screened</td>
<td>amidase</td>
<td><em>B. megaterium</em></td>
<td>Bs WB800</td>
<td>3.58-fold greater activity than control (pBSH1)</td>
<td>[130]</td>
</tr>
<tr>
<td>P43–P$_{laps}$</td>
<td>-</td>
<td>pBE980a</td>
<td>OE due to dual promoter</td>
<td>2,3-BD, TTMP, acetoin</td>
<td>Bs BS2</td>
<td>Bs BS2</td>
<td>36.4% more BD, 36.7% more acetoin, and 95.5% more TTMP vs. single $P_{laps}$/P$_{43}$</td>
<td>[85]</td>
</tr>
<tr>
<td>P$<em>{hpaII}$–P$</em>{amyQ}$</td>
<td>SPamyQ</td>
<td>pHYCGT1</td>
<td>multiple deletions (srfC, spoIIAC, nprE, aprE, amyE)</td>
<td>$\beta$-CGTase (r)</td>
<td>-</td>
<td>Bs CCTCC M 2016536</td>
<td>20% higher expression than $P_{amyQ}$ (&gt;2.4-fold increase compared to 7 other promoters)</td>
<td>[131]</td>
</tr>
<tr>
<td>P$<em>{gsb}$–P$</em>{hpaII}$</td>
<td>SPYncM</td>
<td>pBSG11 (pMA5-BSAP)</td>
<td>6 fusion promoters compared SP library screening</td>
<td>aminopeptidase (r)</td>
<td>-</td>
<td>Bs WB600</td>
<td>&gt;2-fold higher EA than the single promoters; &lt;20% increase with SP$_{YncM}$</td>
<td>[132]</td>
</tr>
<tr>
<td>P43–P$_{hpaII}$</td>
<td>-</td>
<td>pUB110</td>
<td><em>dal</em> KO in <em>Bs</em> chromosome via cre/Lox recombination</td>
<td>D-psicose 3-epimerase</td>
<td>Clostridium scindens 35704</td>
<td>Bs 1A751</td>
<td>20–30% higher EA than the $P_{hpaII}$</td>
<td>[133]</td>
</tr>
</tbody>
</table>

OE = Overexpression; KO = Knockout. 2 (r) = reporter; 2,3-BD = 2,3-butanediol; TTMP = Tetramethylpyrazine; BMP = Beefy Meaty Peptide; $\beta$-CGTase = $\beta$-cyclodextrin glycosyltransferase. 3 *B. = Bacillus; Bs = Bacillus subtilis; Ps. = Pseudomonas. 4 EA = Enzyme activity.
A rather more impressive result was obtained with \( P_{Bh4} \), a synthetic promoter approximately three times stronger than \( P_{srfA} \) (itself 30% stronger than \( P_{43} \)), from which it was derived via two rounds of consecutive evolution, including primary and secondary promoter mutation libraries (pPMLs, sPMLs). This study found that a 7-bp region immediately upstream of \((-10)\) in the spacer sequence of the parent promoter was critical for promoter strength [125].

IPTG-inducible promoters have also been remodeled in this way. A novel \( P_{grac100} \) promoter was derived from \( P_{grac01} \) via optimization of the UP element \((-44 \text{ to } -37)\) and the regions around \((-35), \ (-15), \ (-10), \) and \(+1)\). Having screened a library of 84 promoters, the authors selected \( P_{grac100} \) as the strongest. 9.2 times higher \( \beta\)-galactosidase activity after induction with 0.1 mM IPTG [126].

Promoters can be combined with various functional units to modify the expression from constitutive to inducible. A combination with a synthetic riboswitch produced P43′-riboE1, a first cousin of dual promoters (see Section 4.2.3) able to switch from constitutive to inducible expression under the influence of 4 mM theophylline. Remarkably, the induced expression was dose-dependent and consistently higher than single constitutive promoters such as \( P_{srfA}, P_{aprE}, \) and \( P_{43} \) [127]. Nanobodies derived from single-chain-antibodies of the Camelidae family (camels and llamas) were expressed in \( B. \) subtilis WB800N under the control of the strong promoter \( P_{avrE} \), which, however, was first converted from constitutive to inducible by the addition of the lac operator from \( E. \) coli [128].

4.2.3. Fusion Promoters

Fusion promoters provide constitutive, non-inducible, and highly efficient expression. Usually, two promoters are fused to produce a dual promoter with higher activity. This cumulative effect does not seem to extend further. One comparison of nine dual and five triple promoters showed consistently lower activity of the latter, at least 20 percent on average [134]. Dual promoters can be tedious and time-consuming to produce, and sometimes one must wonder whether the effort is worth it. One study screened 33 promoters, of which only six were chosen (\( P_{ylbr}, P_{spsVG}, P_{aprE}, P_{glyD}, P_{fH41}, \) and \( P_{amyE} \)) for their higher expression than the original \( P_{cdd}(P_{43}) \). The best dual promoter, \( P_{amyE-cdd} \), combined with \( SP_{pac} \) conferred a 3.58-fold increase in amidase activity compared to the control pBSH1 vector [130]. Five single promoters, \( P_{grac}, P_{43}, P_{gsiP}, P_{luxS}, \) and \( P_{aprE} \), were inserted downstream from \( P_{laps} \). Of the six dual promoters obtained (including \( P_{laps}^{-}P_{laps}^{II}, P_{gsiP}^{-}P_{laps}^{II} \) showed the strongest enzyme activity (aminopeptidase as a reporter gene)—73 U/mL; about a 2-fold increase; expectedly; compared to the single promoters; \( P_{laps}^{-} \)and \( P_{gsiP}^{-} \): both showing 32–33 U/mL. The same study also tested 19 SP, but even the best of them (\( SP_{Yrich} \)) produced only a 1.2-fold increase over the native \( SP_{ap} \)—89 U/mL; or not even a 20% increase compared to the vector without a signal peptide [132].

On the other hand, dual promoters can produce startling effects with minimal effort. Simple RBS modification proved spectacularly successful in the case of the dual promoter \( P_{laps}^{-}P_{gb} \)—more than 130 times higher activity than the wild type in shake-flasks and another 1.7 times increase in the 5 L fermenter. Three vectors (pP43NMK, pMA0911, pSTOP1622), four singles (\( P_{gb}, P_{Bh4}, P_{43}, P_{laps} \)), seven dual promoters, and seven RBS modifications were investigated in that study [129]. Simple overexpression of 2,3-butanediol dehydrogenase (BDH, encoded by \( bdhA \)) under the control of the dual promoter \( P_{43}^{-}P_{laps} \) in \( B. \) subtilis BS2, aided by optimization of the metabolic conditions, led to 36.4% more butanediol, 36.7% more acetoin, and 95.5% more TTMP (Tetramethylpyrazine) compared to \( P_{laps} \) or \( P_{43} \) alone [85].

Occasionally, dual promoters require elaborate genetic engineering quite apart from their construction. \( P_{laps}^{-}P_{amyQ} \) produced the highest extracellular \( \beta\)-CGTase (\( \beta\)-cyclodextrin glycosyltransferase) activity (30.5 U/mL) from six dual promoters, but that was only about 20% higher than the \( P_{amyQ} \) (24.1 U/mL), which in turn showed at least 2.4-fold higher activity compared to seven single promoters (\( P_{srf}, P_{sps}, P_{gsiP}, P_{laps}, P_{aprE}, P_{gb} \), and \( P_{amyQ} \)), the apostrophe apparently indicating a difference of species: \( P_{amyQ} \) from \( B. \) amyloliq-
uefaciens, PamyQ from B. subtilis; Pxyf from B. megatherium, Pxyf from B. subtilis). B. subtilis CCTCC M 2016536, a strain with five genes deleted (srfC, spoIAC, nprE, aprE, and amyE) for more robust protein expression, was used to test the industrial promise of PhpaII–PamyQ, and it produced a remarkable 571.2 U/mL in a 3 L fermenter [131]. D-psicose 3-epimerase (DPEase), a curious enzyme engaged in catalyzing the epimerization of D-fructose to D-allulose (a rare sugar, 70% as sweet as sucrose), was cloned from Clostridium scindens 35704 into B. subtilis 1A571 to engineer a strain producing food-grade DPEase. The dual promoter P43–PampaII, obtained by cloning P43 into pUB110, produced only 20–30% higher activity. The dal gene for D-alanine racemase was knocked out via Cre/Lox recombination in order to exchange antibiotic resistance for alanine as a selective marker [133].

4.3. Vector Engineering in Bacillus spp.

4.3.1. Vector Remodeling

A novel salt-inducible plasmid (pSaltExSePR5) was constructed based on pLPB9 from L. plantarum BCC9546, originally isolated from Thai fermented sausage (opuAA promoter). Protease from Halobacillus sp. SR5-3 as a reporter gene estimated 70-fold higher activity with 4 M NaCl compared to non-induced culture (Table 5) [135]. Novel vector constructs based on pHT01 (for trs expression) and pIEFBPR (for deletion of 6 genes), plus promoter manipulation (PgroES substituted with P43), different signal peptides (SPphoD exchanged for SPYubh), and knockout of at least 6 different genes (involved in autolysis and maltose transport) finally produced a 10-fold increase in extracellular trehalose synthase, an enzyme capable of converting maltose to trehalose in a single step [136]. Eight shuttle vectors were constructed based on pWB980 and tested with alkaline protease (spro1) and pectate lyase (pelN) as reporters. Different insertion sites for ori from E. coli and deletion of bleoR (an unnecessary selective gene for bleomycin) further improved the copy number, finally reaching 550–600. The overall increase in lyase and protease activities was 2.5–3 times [137]. Microbial transglutaminase (MTG) from Streptomyces mobaraensis, a crosslinking enzyme that improves protein stability, was tested with two combinations of promoter and signal peptide, SPwapA from B. subtilis 168 with the constitutive PampaII, and SPamyQ from B. amyloliquefaciens with the inducible Plgc. MTG concentration showed a 10–15% fluctuation due to the SP but, curiously, no appreciable difference in the enzymatic activity [138]. An inventive combination of the strong promoter PgsrE (stronger than PgroES, Pveg, P43, and PtrnQ), non-canonical amino acid (ncAA) incorporation for site-specific protein secretion, and the addition of the trpa-terminator to the 3’end and lacO-stem-loop to the 5’end of the reporter gene, all realized in the expression plasmid pLIKE, finally produced a 10-fold increase in the GFP expression. The system was verified with MAK33-1VL, the variable light chain domain of the murine monoclonal antibody against human muscle creatine kinase (hmCK) [139].

4.3.2. Promoter and Signal Peptide Screening

Large-scale screening of signal peptides and promoters has produced some intriguing results. No fewer than 73 signal peptides from B. subtilis 1A747 and B. subtilis 168 were cloned into the vector pWBPRO1 (constructed based on the high-copy pWB980; 121 copies/cell) and screened with alkaline serine protease as a reporter gene. They produced activities that ranged more than 40 times, from 21 U/mg to 953 U/mg for SPDacB. Nine dual and five triple promoters were tested in the same study, all of them based on various permutations of P43, Pshuttle-069, Pshamy, Pbaamy, and Phcapr. Pbaamy–Pbaamy, comprising the α-amylase promoters from B. subtilis and B. amyloliquefaciens, triggered the highest protease activity—a 3.7-fold increase [134]. Six signal peptides (SPamyL, SPlipA, SPnprB, SPnprE, SPphoD, and SPYubh) and five promoters (PampaII, PaprE, P43, PnprL, and Pmglg—this last maltose-inducible, constructed in the same lab but previously unpublished) were tested with the manB gene (β-mannanase) from B. licheniformis DSM13. Pmglg produced 3-fold higher enzyme activity than PampaII, while SPlipA was twice as efficient as SPnprB [140].
Table 5. Vector engineering in *Bacillus* spp.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Signal Peptide</th>
<th>Vector</th>
<th>Genetic Modifications</th>
<th>Target</th>
<th>Source</th>
<th>Host</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_{ops/AA}</td>
<td>SP_{sa}</td>
<td>pSaltExSePR5</td>
<td>new vector with a salt-inducible promoter</td>
<td>protease</td>
<td><em>Halobacillus</em> sp. SR5-3</td>
<td>Bs WB800</td>
<td>70-fold higher protease activity with 4 M NaCl than the non-induced culture</td>
<td>[135]</td>
</tr>
<tr>
<td>P_{43}</td>
<td>-</td>
<td>pUC980</td>
<td>pUC19 ori inserted into pWB980, bleoR deletion</td>
<td>alkaline protease; pectate lyase</td>
<td><em>Bacillus</em> sp. 221, <em>Paenibacillus</em> sp. 0602, <em>Anoxybacillus</em> sp. LM18-11</td>
<td>Bs WB600</td>
<td>2.5–3 times higher activity than pWB980 constructs for <em>pelN1</em> and <em>spro1</em></td>
<td>[137]</td>
</tr>
<tr>
<td>P_{43}</td>
<td>SP_{YwbN}</td>
<td>pHT01 pIEFBPR</td>
<td>P_{grac} discarded; 6 genes KO (xP, skfA, ltyC, sdpC, malP, amyE); SP_{phoD} exchanged for SP_{YwbN}</td>
<td>trehalose synthase</td>
<td>-</td>
<td>Bs WB800N</td>
<td>about 10-fold increased activity overall</td>
<td>[136]</td>
</tr>
<tr>
<td>P_{mgb}</td>
<td>SP_{lipA}</td>
<td>pMA5</td>
<td>6 SP and 4 promoters were cloned and tested</td>
<td>β-mannanase</td>
<td><em>B. licheniformis</em> DSM13</td>
<td>Bs 1A751</td>
<td>2-fold higher EA than least efficient (SP_{aprE}); 3-fold higher EA than P_{hpaII}</td>
<td>[140]</td>
</tr>
<tr>
<td>P_{hpaII}</td>
<td>SP_{amyA} SP_{amyQ}</td>
<td>pHT43 pMA5</td>
<td>Inducible P_{lac} used for SP_{amyQ}</td>
<td>MTG</td>
<td><em>Str. mobaraensis</em> CGMCC 4.5591</td>
<td>Bs 168 Bs WB600</td>
<td>63 mg/L MTG with SP_{amyA}; 10–15% less with SP_{amyQ}; almost no difference in enzymatic activity</td>
<td>[138]</td>
</tr>
<tr>
<td>P_{aprE}</td>
<td>SP_{nprE}</td>
<td>pMA5 pDL</td>
<td>PrsA lipoprotein OE; 6 SP tested</td>
<td>amylase</td>
<td><em>B. licheniformis</em> CICC 10181</td>
<td>Bs 1A751</td>
<td>2.5-fold overall increase</td>
<td>[141]</td>
</tr>
<tr>
<td>P_{grpD}</td>
<td>SP_{lspB}</td>
<td>pWB980</td>
<td>pro-peptide from <em>S. hygroscopicus</em></td>
<td>MTG</td>
<td><em>Str. mobaraensis</em></td>
<td>Bs WB600</td>
<td>&gt;20% higher EA compared to P_{43}</td>
<td>[142]</td>
</tr>
<tr>
<td>T7</td>
<td>SP_{synD} (lypo type)</td>
<td>pDMT pDBT</td>
<td>24 SP tested; nprB and mpr KO; hEGF cassette integrated into nprB</td>
<td>hEGF</td>
<td>Homo sapiens</td>
<td>Bs PT5 Bs PT6 Bs PT7</td>
<td>almost 2-fold increase SP_{synD}; only 6 of 24 SP guided hEGF into extracellular space</td>
<td>[93]</td>
</tr>
<tr>
<td>P_{Baamy}</td>
<td>SP_{DacB}</td>
<td>pWBPRO1</td>
<td>72 SP, 9 dual, and 5 triple promoters were screened</td>
<td>alkaline serine protease (r)</td>
<td><em>B. clausii</em></td>
<td>Bs WB600</td>
<td>3.7-fold increase with SP_{DacB} and P_{Baamy-P_{Baamy}}</td>
<td>[134]</td>
</tr>
<tr>
<td>Promoter</td>
<td>Signal Peptide</td>
<td>Vector</td>
<td>Genetic Modifications ¹</td>
<td>Target ²</td>
<td>Source ³</td>
<td>Host ³</td>
<td>Effect ⁴</td>
<td>Reference</td>
</tr>
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<td>----------</td>
</tr>
<tr>
<td>P&lt;sub&gt;groES&lt;/sub&gt;</td>
<td>SP&lt;sub&gt;amyE&lt;/sub&gt;</td>
<td>pLIKE</td>
<td>trpA-terminator to the 3′ end and lacO-stem-loop to the 5′ end of the reporter gene</td>
<td>MAK33-VL</td>
<td>-</td>
<td>Bs K7, Bs PG10</td>
<td>10-fold increased expression with GFP; verified with MAK33-L</td>
<td>[139]</td>
</tr>
<tr>
<td>P&lt;sub&gt;43&lt;/sub&gt;</td>
<td>-</td>
<td>pHY300 T2(2)-ori</td>
<td>Δepr, ΔaprA, Δmpr, ΔaprE, Δmpr, ΔhprA, ΔbacABC; aprN inserted</td>
<td>nattokinase</td>
<td>Bs 168</td>
<td>Bl DW2</td>
<td>25.7% higher EA in the strain with 7 deletions</td>
<td>[143]</td>
</tr>
<tr>
<td>P&lt;sub&gt;43&lt;/sub&gt;</td>
<td>-</td>
<td>pBSCas9 PHP13</td>
<td>multiplex genome editing; ribA, ribB, and ribH engineered for improved riboflavin production</td>
<td>riboflavin</td>
<td>-</td>
<td>Bs BS89</td>
<td>80% success in 1–8 kb deletions &gt;90% success in 1–2 kb insertions 100% site-directed mutagenesis</td>
<td>[144]</td>
</tr>
<tr>
<td>P&lt;sub&gt;grac&lt;/sub&gt;</td>
<td>SP&lt;sub&gt;npr&lt;/sub&gt;</td>
<td>pHT01 pDR-sgRNA</td>
<td>KO epsA-O, cysO, sacB; OE CscA, SacC, OsC introduced</td>
<td>γ-PGA</td>
<td>Ps. mucidolens Bl 14580</td>
<td>Ba NB</td>
<td>32% more γ-PGA</td>
<td>[145]</td>
</tr>
</tbody>
</table>

¹ OE = overexpression; KO = knockout; SP = signal peptide. ² (r) = reporter; MTG = microbial transglutaminase; γ-PGA = poly-γ-glutamic acid; hEGF = human epidermal growth factor. ³ B. = Bacillus; Bs = Bacillus subtilis; Bl = Bacillus licheniformis; Ba = Bacillus amyloliquefaciens; Ps. = Pseudomonas; Str. = Streptomyces. ⁴ EA = Enzyme activity.
Of the 24 SP tested, only six proved capable of guiding hEGF into the medium at all, with the most efficient of them (P<sub>xynD</sub>) reaching 320 mg/L in B. subtilis PT7. Additional hEGF cassettes integrated into the nprB gene raised the concentration to only 12% (360 mg/L), another telling reminder of how important signal peptides are [93]. Four promoters (P<sub>pyl</sub>, widely used from Streptococcus aureus; P<sub>43</sub>, P<sub>Ave</sub>, strong constitutive promoters from B. subtilis; and P<sub>amyL</sub>, native promoter from B. licheniformis) and six signal peptides (SP<sub>nprE</sub>, SP<sub>paeE</sub>, SP<sub>AmyL</sub>, SP<sub>UapA</sub>, SP<sub>gycM</sub>, SP<sub>amyE</sub>, and SP<sub>sacB</sub>) were tested with the amyL gene from B. licheniformis CICC 10181 cloned into B. subtilis 1A751. Overexpression of the PsA lipoprotein, a folding factor for exoproteins from the family of parvulin-type PPLases, contributed to a 2.5-fold overall increase in amylase activity [141].

4.3.3. CRISPR/Cas9 Genome Editing

In recent years, the CRISPR/Cas9 system has emerged as a state-of-the-art genome editing tool that is efficient, versatile, and easy to use. In B. licheniformis DW2, the CRISPR-Cas9 technique was used to construct a strain with multiple deletions, among them the 42.7 kb bacitracin synthase cluster (bacABC), which was deleted with 79% efficiency. However, other gene disruptions ranged from 100% to less than 12% in efficiency, and they all combined produced only 25.7% higher activity of heterologous nattokinase inserted as a reporter gene [143]. Multiplex genome editing with CRISPR-Cas9 in B. subtilis achieved at least 80% efficiency in 1–8 kb gene deletions, at least 90% efficiency in insertions of 1–2 kb, nearly 100% efficiency in site-directed mutagenesis, 23.6% efficiency in the deletion of large DNA fragments, and nearly 50% efficiency for three simultaneous point mutations. The system was tested with fine-tuning of three genes from the riboflavin operon (ribA, ribB, and ribH), and 111 out of 190 strains from a B. subtilis library were improved in terms of riboflavin production [144]. In B. amyloliquefaciens NB, the CRISPR-Cas9n system was used to enhance the production of poly-γ-glutamic (γ-PGA) acid from inulin. The native inulin hydrolase CscA was overexpressed, while exo-type levanase (SacC) from B. licheniformis 14580 and endo-inulinase (OsC) from Pseudomonas mucidolens were introduced into B. amyloliquefaciens NB to further increase its inulin utilization ability. Furthermore, the polysaccharide operon epsA-O and the γ-PGA hydrolase encoded by culO were deleted to minimize the formation of byproducts and the depletion of the main product, respectively [145].

5. Metabolic Engineering in Bacillus spp.

5.1. Manipulation of Metabolic and Secretory Pathways

Sophisticated manipulation of metabolic pathways has been explored for the production of many different compounds important enough to afford it (Table 6). Overexpression of NADH oxidase YODC combined with knock-out of bdhA (acetoin reductase) led to the production of 35.3% more acetoin by B. subtilis JNA 3-10. The by-products butanediol, lactic acid, and ethanol were considerably reduced as well, by 92.3%, 70.1%, and 75.0%, respectively. Interestingly, the most pronounced effect was obtained with moderate overexpression of YODC under the promoter P<sub>bdhA</sub> rather than the stronger P<sub>pyl</sub> [146]. The production of γ-PGA, an anionic polymer with applications in medicine, light chemical industry, wastewater treatment, and agriculture, by B. licheniformis WX-02 was achieved via spectacular metabolic rewiring. Overexpression of pdhABCD (pyruvate dehydrogenase) and citA (citrate synthase) strengthened the pyruvate flux into the TCA; deletion of pfdB (pyruvate-formate lyase) prevented pyruvate “leaking” towards formate; and repression of aceA (isocitrate lyase) diminished the glyoxylate shunt. A nearly 70% overall increase was achieved, even though only an ordinary P<sub>43</sub> promoter from B. subtilis 168 was used [147]. 1-deoxynojirimycin (1-DNJ), an efficient α-glucosidase inhibitor with promising application in anything from functional foods to Type-II diabetes medicines, was produced with 33% more by B. amyloliquefaciens HZ-12 with integrated gabT1, gutB1, and glcP. The overexpression of GlcP, a glucose facilitator protein, promoted DNJ synthesis and also reduced by-product acetoin by 36.7% [148]. A tenfold increase in 1-DNJ production (267 mg/L) by the same strain was achieved by weakening the PTS pathway (eliminating the ptsG gene by
homologous recombination), while at the same time strengthening the non-PTS pathway by deleting its repressor $iolR$ [149].

N-acetylglucosamine (GlcNAc), an amino sugar of some importance in healthcare, was produced by $B. subtilis$ BN0-GNA1, a strain previously designed for the purpose but reinforced by sophisticated programming of metabolic pathways. The PTS system was blocked by gene deletions for three subunits ($yzzE$, $ypqE$, and $ptsG$); glucose import and utilization were improved by overexpression of $gicP$ (encoding a sugar transporter) and $gicK$ (encoding glucokinase, the enzyme phosphorylating glucose, the first and rate-limiting step of glycolysis); and finally, codon-optimizing repression of glycolysis, the pentose phosphate pathway, peptidoglycan synthesis, and the TCA. As a result, the GlcNAc titer was almost doubled (6.5 vs. 13.2 g/L) in flasks compared to the original strain; only a 1.72-fold increase, however, was achieved in a 3 L fed-batch bioreactor. Interestingly, nearly half of the total effect (47.6%) was due to the PTS blockage [150].

Metabolic manipulation does not exclude rigorous genetic modification, e.g., gene screening or promoter engineering. No fewer than 15 genes for prephenate dehydrogenase (which catalyzes the synthesis of $p$-hydroxyphenylpyruvate using prephenate and NAD$^+$ as substrates) were screened in order to increase L-tyrosine production by $B. amyloliquefaciens$ HZ-12. $P_{43}$ was compared to 13 other promoters, including one dual ($P_{43}–P_{ylB}$), of different strengths: eight of them it surpassed in a statistically significant way; while there was no significant difference with the other five, $P_{43}$ was nevertheless able to confer greater production of L-tyrosine than any of them (about 400 mg/L). Five different UTRs were also tested, but only one produced a significant difference, and then only 16% (475 mg/L) [151].

Metabolic engineering may be extended to include secretory pathways. Overexpression of 23 Sec pathway components and the PrsA lipoprotein in $B. subtilis$ 1A751 achieved 3.2- and 5.5-fold higher expression of two amylases, AmyL and AmyS, respectively. However, the corresponding increase in enzymatic activity, even after overexpression of the partial $dnaK$ operon and induction by xylose ($P_{xylA}$), was only 60% and 73% [152]. The activity of extracellular lipase LipA, a versatile biocatalyst widely used in industrially relevant bioconversion reactions, was increased 14-fold when a unique $P_{AE}$ promoter, created by ligating the A1 sequence of bacteriophage $\phi 29$ to the mRNA stabilizer of the $aprE$ gene, was used in $B. subtilis$ BNA. Combined overexpression of the Sec pathway components $secDF$ and $prsA$ conferred an additional increase of 59%. It is interesting to note that separate overexpression of $secDF$ and $prsA$ produced a 28% and 49% increase, respectively, in lipase activity [153].

5.2. Cofactors Fine-Tuning

Cofactors are small non-protein molecules vital for the function of many enzymes, either metal ions ($Zn^{2+}$, $Mg^{2+}$, $Fe^{3+}$, $Cu^{2+}$) or small organic compounds existing in both reduced and oxidized forms (NAD$^+$/NADH, NADP$^+$/NADPH, FAD$^+$/FADH). Cofactors are usually synthesized de novo in the bacterial cell; therefore, they constitute a convenient target for metabolic manipulation [1].

Microbial production of riboflavin (vitamin $B_2$), the chief precursor of flavin coenzymes (FMN, FAD), has been the subject of intense metabolic engineering for years. $B. subtilis$ PK, a strain carrying multiple copies of the riboflavin operon, was further improved by promoter exchange ($P_{43}$ for the native $P_{ribP1}$) and overexpression of multiple genes ($purF$, $purM$, $purN$, $purH$, and $purD$) involved in the biosynthesis of GTP, one of the main precursors of riboflavin, from phosphoribosylpyrophosphate, finally achieving a 31% higher titer and a 25% higher yield of riboflavin [154].
### Table 6. Metabolic engineering in *Bacillus* spp.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Vector</th>
<th>Genetic Modifications</th>
<th>Target</th>
<th>Source</th>
<th>Host</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₄₃</td>
<td>T2(2)-Ori</td>
<td>OE (^1) pdhABCD and citA; ΔpflB; repression of aceA</td>
<td>γ-PGA</td>
<td>Bs 168</td>
<td>Bl WX-02</td>
<td>69% higher yield</td>
<td>[147]</td>
</tr>
<tr>
<td>P₁₁₆₆₄</td>
<td>pMA5-PA</td>
<td>ΔβdhA; moderate expression of yodC; P₁₁₆₆₄ exchanged for P₁₁₆₆₄</td>
<td>acetoin</td>
<td>Bs 168</td>
<td>Bs JNA 3-10</td>
<td>35.3% more acetoin; 92.3%, 70.1%, and 75.0% less BD, LA, and EtOH, respectively</td>
<td>[146]</td>
</tr>
<tr>
<td>P₄₃</td>
<td>T2(2)-ori</td>
<td>OE glcP; gabTT and gutB1 integrated; amylL terminator from Bl DW2</td>
<td>1-DNJ</td>
<td>-</td>
<td>Bs HZ-12</td>
<td>33% increased production 36.7% less acetoin by-product</td>
<td>[148]</td>
</tr>
<tr>
<td>P₄₃</td>
<td>T2(2)-ori</td>
<td>ptsG weakened; ΔiolR; promoter change; and 5’-UTR optimizations</td>
<td>1-DNJ</td>
<td>-</td>
<td>Bs HZ-12</td>
<td>10.2-fold higher amount overall</td>
<td>[149]</td>
</tr>
<tr>
<td>P₄₃ P₄₃</td>
<td>p43NMK PDG148</td>
<td>ΔyggE, ΔyppE, ΔptsG; glcP and glcK OE; pathway repression with codon-optimizing strategies</td>
<td>GlcNAc</td>
<td>S. cerevisiae</td>
<td>Bs BN0-GNA1</td>
<td>2-fold higher titer than the original strain in flasks; 1.72-fold more in a 3 L fed-batch bioreactor</td>
<td>[150]</td>
</tr>
<tr>
<td>P₄₃</td>
<td>pHY300PLK</td>
<td>TamyL terminator Bl WX-02; synthetic 5’-UTR; 15 genes for prephenate dehydrogenase screened</td>
<td>L-tyrosine</td>
<td>Ba HZ-12</td>
<td>Ba HZ-12</td>
<td>42% higher yield than the control strain</td>
<td>[151]</td>
</tr>
<tr>
<td>P₄₃ P₄₃</td>
<td>pMA5</td>
<td>OE of 23 genes involved in the Sec pathway, PrsA lipoprotein, partial shuK operon; SP₄₃ and SP₄₃</td>
<td>2 amylases AmyL AmyS</td>
<td>Bl CICC 10181 Go ATCC 31195</td>
<td>Bs 1A751</td>
<td>3.2-fold higher expression for AmyL; 5.5-fold for AmyS; 60 and 73% higher EA</td>
<td>[152]</td>
</tr>
<tr>
<td>P₄₃ P₄₃</td>
<td>pH13</td>
<td>OE of 4 Sec pathway components (secA-prfB, secDF, secYEG, prsA); promoter change</td>
<td>lipase</td>
<td>Bs 168</td>
<td>Bs BNA</td>
<td>14-fold increase in EA compared to P₄₃; further 59% higher with secDF and prsA OE</td>
<td>[153]</td>
</tr>
<tr>
<td>P₄₃</td>
<td>pUCL92</td>
<td>OE purF, purM, purN, purH, purD; promoter exchange</td>
<td>riboflavin</td>
<td>-</td>
<td>Bs PK</td>
<td>31% higher titer, 25% higher yield</td>
<td>[154]</td>
</tr>
<tr>
<td>P₄₃</td>
<td>pSS</td>
<td>mutations RibC (G199D), ribD+ (G+39A) and YvrH (R222Q)</td>
<td>riboflavin</td>
<td>-</td>
<td>Bs 24/pMX45</td>
<td>3.4-fold higher titer than the initial strain; 23.4% increase due to the YvrH mutation</td>
<td>[155]</td>
</tr>
</tbody>
</table>

\(^1\) Of the targets, some are considered for the improved production of a specific compound or pathway repression.
Table 6. Cont.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Vector</th>
<th>Genetic Modifications ¹</th>
<th>Target ²</th>
<th>Source ³</th>
<th>Host ³</th>
<th>Effect ⁴</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_{vegI}</td>
<td>pH13</td>
<td>KO apt, xpt, adeC, nrdE, nrdF</td>
<td>riboflavin</td>
<td>-</td>
<td>Bs 168</td>
<td>41.50% higher production in ΔadeC mutants; 13.12% increase with RNR repressed</td>
<td>[156]</td>
</tr>
<tr>
<td>P_{bdhA}</td>
<td>pMA5</td>
<td>OE dhaD, gldA, acr introduction of ALSR</td>
<td>2,3-BD</td>
<td>K. pneumoniae ATCC 25955</td>
<td>Ba B10-127</td>
<td>102.3 g/L; 1.16 g/L/h</td>
<td>[92]</td>
</tr>
<tr>
<td>Pr43</td>
<td>T2(2)-Ori</td>
<td>OE zwf, pyk, argA; ΔargF, ΔahrC; TamyL terminator B/ WX-02</td>
<td>putrescine</td>
<td>E. coli</td>
<td>Ba HZ-12</td>
<td>5.51 g/L, 0.11 g/L/h, and 0.14 g/g, with xylose as substrate</td>
<td>[157]</td>
</tr>
<tr>
<td>Pr43</td>
<td>pP43NMK PDG148</td>
<td>KO pyk, kdgA, ywKA, pckA, ytsf; melA, matS; OE pycA, pfaA, fbaA</td>
<td>GlcNAc</td>
<td>S. cerevisiae Bs 168</td>
<td>A. flocculosa</td>
<td>3.7-fold higher titer, 4-fold higher yield, and 1.6-fold higher productivity than the initial strain</td>
<td>[158]</td>
</tr>
</tbody>
</table>

¹ OE = Overexpression; KO = Knockout; Bl = Bacillus licheniformis. ² 2,3-BD = 2,3-butanediol; 1-DNJ = 1-deoxynojirimycin; GlcNAc = N-acetylglucosamine; γ-PGA = poly-γ-glutamic acid. ³ B. = Bacillus; Bs = Bacillus subtilis; Ba = Bacillus amyloliquefaciens; Ps. = Pseudomonas; Gs. = Geobacillus stearothermophilus; K. = Klebsiella; A. = Anthracocystis. ⁴ EA = Enzyme activity; RNR = ribonucleotide reductase; BD = 2,3-butanediol; LA = Lactic acid; EtOH = ethanol.
Riboflavin is negatively regulated by FMN via the so-called RFN element (ribO), a region of 300 base pairs upstream of the first gene in the rib operon. One ingenious study achieved more than 50-fold increased expression of the rib operon in *B. subtilis* 24/pMX45 by two specific mutations, RibC (G199D) and ribD+ (G39+A), which led to more than three times higher production of riboflavin, from 50 mg/L to more than 170 mg/L, by reducing the FMN pool and thus effectively upregulating the rib operon [155]. Knock-out of adeC, encoding an enzyme (adenine deaminase) from the interconversion pathways of purine metabolism, increased riboflavin production by almost 42%, chiefly because of the increased amount of GTP. The same study also explored the salvage pathways and the ribonucleotide reductase (RNR) but found them of lesser importance; knocking out genes involved in the salvage of adenine (apt) and xanthine (xpt) nucleotides, as well as several RNR genes (rnrE, rnrF), increased riboflavin production by no more than 14% [156].

Cofactors can be manipulated in subtle ways in order to influence the microbial production of various useful compounds, for instance, platform chemicals. Production of 2,3-BD from biodiesel-derived glycerol in *B. amyloliquefaciens* was increased, with a corresponding decrease in by-products, by the introduction of a cofactor regeneration system, which ensured that NAD+ was reduced to NADH more effectively. Overexpression of two glycerol dehydrogenases from *K. pneumoniae*, DhaD and GldA, and acetoine reductase (ACR), plus the introduction of the transcriptional regulator ALSR under the control of the moderate promoter PbdhA, finally yielded 102.3 g/L 2,3-BD with a productivity of 1.16 g/L/h [92]. Production of putrescine, an exotic C4 platform chemical, by *B. amyloliquefaciens* HZ-12 was improved by modular engineering that optimized the supply of NADH and ATP and overexpression of glucose-6-phosphate dehydrogenase (zwf) and pyruvate kinase (pyk). Additionally, an ornithine decarboxylase introduced from *E. coli* (specC, specF), deletion of argF (ornithine carbamoyltransferase) and ahrC (arginine repressor), and overexpression of argA (N-acetylglutamate synthase) constituted another module designed to secure the production of putrescin from ornithine. Combined, putrescin production reached 5.51 g/L, 0.11 g/L/h, and 0.14 g/g from xylose [157].

Four synthetic NAD(P)-independent routes were introduced in order to improve the titer, yield, and productivity of a strain of *B. subtilis* designed as a super-producer of GlcNAc. The key enzyme (GNA1) that acetylates glucoseamine-6-phosphate is missing in *B. subtilis* and was introduced from *S. cerevisiae*. Furthermore, deletion of pyk and kdgA reduced the formation of pyruvate from PEP and through the pentose phosphate pathway; PEP accumulation was prevented by deletion of pckA, which blocked gluconeogenesis from oxaloacetate to PEP, and substitution of the weak native promoters of pfkA and fbaA with the strong P43 promoter, which resulted in increased amounts of fructose-6-phosphate, one of the major substrates for the formation of GlcNAc; pyruvate flux was directed toward the TCA, instead towards byproducts like acetoin and butanediol, via overexpression of pycA, encoding pyruvate decarboxylase. As a result of this spectacular metabolic engineering, the GlcNAc titer in shake flasks was increased 3.7-fold, the yield 4-fold, and the productivity 1.6-fold [158].

6. Conclusions

Bioengineering in *Bacillus* spp. has changed beyond recognition in the last few decades. The genetic improvement of the strains involves the development of a series of molecular methods for their application, such as creating vectors, targeting knockout genes to establish alternative metabolic pathways, introducing heterologous genetic information to confer new traits, and introducing new methods of cell transformation. However, when *Bacillus* spp. is engaged in gene cloning, some difficulties specific to Gram-positive hosts must be considered. These are, for example, the small number of copies of autonomously replicating vectors (and the stringent copy-number control), their structural and segregational instability, and a comparatively smaller number of transformants obtained. However, these challenges lead to brainstorming and cutting-edge experiments. Thus, a large number of integrative vectors and alternative routes for introducing the heterologous constructs...
into the *Bacillus* cell have been developed. There has been tremendous progress in the study of promoters and, in general, all mechanisms of control over gene expression and the secretion of recombinant proteins with various applications. Future prospects clearly show that *Bacillus* spp. will continue to be used as an indispensable microbial factory for valuable products, especially under the current conditions of enforcing a circular economy and valuing biomass. Therefore, simultaneous advances in fundamental and applied knowledge of their complex genetic and biochemical machinery are sure to continue in the future.

**Author Contributions:** Conceptualization, A.A. and P.P.; investigation, N.A.; software, E.G.; resources, K.P.; writing—original draft preparation, A.A., N.A. and E.G.; writing—review and editing, P.P. and K.P.; project administration, K.P. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** No new data were created or analyzed in this study.

**Conflicts of Interest:** The authors declare no conflict of interest.

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