Current development in genetic engineering strategies of *Bacillus* species

Huina Dong¹,² and Dawei Zhang¹,²,³*

**Abstract**

The complete sequencing and annotation of the genomes of industrially-important *Bacillus* species has enhanced our understanding of their properties, and allowed advances in genetic manipulations in other *Bacillus* species. Post-genomic studies require simple and highly efficient tools to enable genetic manipulation. Here, we summarize the recent progress in genetic engineering strategies for *Bacillus* species. We review the available genetic tools that have been developed in *Bacillus* species, as well as methods developed in other species that may also be applicable in *Bacillus*. Furthermore, we address the limitations and challenges of the existing methods, and discuss the future research prospects in developing novel and useful tools for genetic modification of *Bacillus* species.

**Keywords:** *Bacillus* species, Genetic engineering strategies, Counter-selection marker, Operator-repressor system, Toxin gene, Site-specific recombination

**Introduction**

*Bacillus subtilis* and some related *Bacillus* species are non-pathogenic, free of exotoxins and endotoxins, and have a recognized history of safe use in foods. These species are also useful for fermentation and large-scale cultivation. Despite these natural advantages, protocols using *Bacillus* species lag far behind those for *Escherichia coli* and *Saccharomyces cerevisiae*, which are the most widely used cellular factories for producing industrially-important enzymes and biochemicals [1,2]. Therefore, efficient genetic manipulation and highly developed systems-level strategies for developing *Bacillus* species as microbial cell factories are needed. Recently, *Bacillus* species have received increased attention for their use in genetic engineering and production of heterologous proteins [3], valuable enzymes, vitamins, platform chemicals, and antibiotics [4-6].

The complete genome sequences of several *B. subtilis* strains have been determined and annotated, stimulating novel methods for interpreting metabolic pathways and supplying an overview of protein machinery [7]. The completion of the genome sequences of industrially-important *Bacillus* species (*Bacillus licheniformis*, GenBank accession number CP000560 and *Bacillus amylo liquefaciens*, GenBank accession number AE017333) revealed a high degree of homology to *B. subtilis* [8-10]. These complete genome sequences not only enhance our understanding of these strains, but also allow advances in genetic manipulations in other *Bacillus* species [6]. With rapid developments in post-genomic studies, simple and efficient genetic tools are required to conveniently enable multiple modifications of the genomes of *Bacillus* species.

Classical chromosomal modification is based on the insertion of a selectable marker, usually a drug resistance gene, into the chromosome of a bacterial strain [11]. Using this strategy, a second selective marker gene is required to introduce another chromosomal modification, so the number of available selection genes limits the feasibility of multiple chromosomal modifications. Moreover, the selectable gene should be removed by single-crossover recombination if the strain is to be used for further genetic manipulation. In addition, the chance of obtaining a positive strain is relatively low, and the selection process is laborious. To overcome these problems, methods that can eliminate marker cassettes in the primary transformants are desperately needed.

Recently, useful tools for genetic modification of *Bacillus* species have emerged from the fields of systems and
synthetic biology. Thus, it is necessary to summarize recent progress, current obstacles, and future goals to inspire more research interest and advance studies in this field. First, we summarize the progress in genetic modification strategies of *Bacillus* species, including several kinds of marker-free genetic modification methods and site-specific recombination strategies. Next, we discuss some strategies developed in other species that could also be used in *Bacillus* species. Lastly, we compare current genetic engineering strategies, analyze their challenges and limitations, and discuss future research goals for developing novel and useful tools for genetic modification of *Bacillus* species.

**Operator-repressor system-based genetic engineering strategies**

The development of various inducible promoter systems has played an important role in the analysis of gene expression and function. Promoters responsive to an assortment of inducing agents, including heavy metals, hormones, and heat shock, as well as several viral, cellular, and bacterial regulatory factors, have been successfully used to manipulate gene expression. Some systems have been applied to genetically engineer *Bacillus* species, and the selection and counter-selection marker cassettes are shown in Figure 1.

### cl as a counter-selection marker

The CI repressor gene (*cI857* or *cl*) from *E. coli* bacteriophage lambda encodes the CI repressor, which can bind to the Pr promoter of lambda phage to suppress its promoter activity. Itaya [12] developed a counter-selection method in which a neomycin-resistance gene is regulated by Pr. This system, controlled by the presence/absence of the CI repressor, allows precise selection for marker-free genetic modification of the chromosome [13-15]. Similarly, Uotsu-Tomita *et al.* [16] developed a double cl-P$_r$ method for positive selection of *B. subtilis* recombinants, and Tsuge *et al.* [17] obtained markerless deletion of *sfp*, *degQ*, and *ppsABCDE* using the cl-P$_r$ system. This method can be repeatedly used by changing the location of the cl gene [18].

**araR as a counter-selection marker**

*araR* encodes a negative regulator of the *ara* operon that can be induced by L-arabinose in *B. subtilis* [19]. Liu *et al.* [20] developed a method in which the chromosomal *araR* locus was replaced by P$_{ara-neo}$, which confers neomycin resistance. By adding L-arabinose to the growth medium, the expression of P$_{ara-neo}$ can be induced based on the presence of *araR* in the chromosome. First, the selection marker cassette cat-araR is inserted upstream of the target gene via recombination, and then selected for with chloramphenicol. Next, single-crossover recombination between two downstream regions removes the cat-araR cassette, together with the target gene. The remaining P$_{ara-neo}$ in the genome can be used again for further genetic modification. The authors obtained 3.8-kb and 41.8-kb deletion strains using this system that left no selective marker gene in the targeted loci.

**Pyrimidine metabolism-based genetic engineering strategies**

Common approaches for counter-selection exploit genes involved in purine or pyrimidine metabolism and are based on the fact that purine or pyrimidine analogs can be converted to toxic compounds. Plating cells on media containing the analog leads to a strong selection for clones that have lost the chromosomally-integrated copy of the gene encoding the converting enzyme. Therefore, parental strains used for genome modification must lack the respective gene for purine or pyrimidine nucleotide biosynthesis. Recently, *upp* and *pyrF*, encoding uracil
phosphoribosyltransferase (UPRTase) and orotidine 5′-phosphate decarboxylase (OMPdecase), respectively, have been used as counter-selection markers in Bacillus species. The relevant pyrimidine metabolism pathway and mechanisms of action of pyrimidine analogs are shown in Figure 2.

**upp as a counter-selection marker**

UPRTase catalyzes the key reaction of the pyrimidine salvage pathway, from uracil to UMP, in many microorganisms. The toxic pyrimidine analog 5-fluorouracil (5-FU) can be converted to 5-fluoro-UMP by UPRTase. The latter compound can be further catalyzed into 5-FU (5-FU) can be converted to 5-fluoro-UMP by UPRTase. The latter compound can be further catalyzed into 5-FU (5-fluoro-dUMP), which is a strong inhibitor of thymidylate synthetase. Deletion of **upp** endows the mutant strain with resistance to 5-FU.

Fabret et al. [21] developed a method using **upp** as a screening marker in *B. subtilis*. A PCR-generated DNA fragment, which consists of the target gene with a desired mutation linked to a **upp** cassette, was inserted into the genome by double-crossover recombination and selected for based on pheomycin resistance. The **upp** gene was excised through recombination of the direct repeats (DR) flanking the **upp** cassette in the linear DNA, and 5-FU-containing medium was used to select for strains that contained the desired chromosomal mutation.

The **upp** cassette has also been used to functionally analyze *B. subtilis* genes by constructing a mutant library [22], point mutations [23], and gene-null strains [24,25]. Morimoto et al. [26] generated a genome-reduced *B. subtilis* strain, MGB874, using the **upp** cassette to sequentially knock out genes. Compared with the parental *B. subtilis* 168 genome, the genome of strain MGB874 is depleted by 874 kb (20.7%), including 865 genes.

Tanaka et al. [27] developed an improved counter-selection system that combines the **upp** and cl-P systems. In this system, the master strain (MS) TF8A Δ**upp**: λPr-neo was constructed by replacing **upp** with a λPr-neo cassette, the counter-selection marker mentioned above. The **upp-phleo-cl** cassette undergoes homologous replacement with a targeted chromosome region after being introduced into the MS. Positive selection for cassette integration is based on resistance to phleomycin. The **upp** gene and the sak promoter construct P sak-λcl are used for counter-selection and cassette removal.

Recently, Shi et al. [28] developed a method combining **upp** and double-strand break (DSB) repair, which is caused by exogenous endonuclease I-SceI and comK overexpression, for fast preparation of competent cells. First, a foreign dsDNA fragment is integrated into the chromosome via double-crossover. The **upp** cassette can then be excised by a second intramolecular homologous recombination. The DSB repair potently induces the second intramolecular recombination, which enhances the frequency of resolution by one to two orders of magnitude.

A method based on **upp** has also been used in *Bacillus* species other than *B. subtilis*. Wemhoff et al. [29] developed an **upp**-based deletion method for *Bacillus pumilus* in which master strains used for gene deletion are generated by targeted deletion of a set of genes, including the essential sporulation gene *yqfD*, enabling rapid allelic exchange between **upp** and *hsdR*. The *hsdR* gene encodes the restrictase of a type I restriction modification system, and its deletion makes a strain readily transformable. The resultant *B. pumilus* mutant is easily transformable with plasmid DNA isolated from *E. coli* strains. In addition, direct gene disruption is possible, which enables relatively rapid genetic manipulations.

**pyrF as a counter-selection marker**

Orotate phosphoribosyltransferase (OPRTase) and OMPdecase, encoded by **pyrE** and **pyrF** respectively, are essential enzymes for metabolism of orotic acid to UMP and 5-fluoroorotate to 5-fluoro-UMP. 5-fluoro-UMP can be further converted into the toxic metabolite 5-fluoro-dUMP. The PyrR protein (encoded by **pyrR**) is an mRNA-binding attenuator, which regulates expression of pyrimidine biosynthetic (**pyr**) genes by sensing UMP or UTP.

Suzuki et al. [30] established a counter-selection system based on deletion of **pyrR** and **pyrF** in *Geobacillus kaustophilus* HTA426. The disruption of **pyrF** and **pyrR** makes the MS auxotrophic for uracil and resistant to 5-
fluoroorotate. Heterologous β-galactosidase and α-amylase genes were integrated in the genome of *G. kaustophilus* by *pyrF*-based counter-selection using pGAM plasmids, without leaving the marker in the target loci. This system may be applied to other organisms harboring *pyrF*, such as *Bacillus*-related bacteria.

**Auxotrophy-based genetic engineering strategies**

**A lysine-auxotrophic strain combined with an operator-repressor system**

The *lysA* gene of *B. subtilis* 168 encodes diaminopimelate decarboxylase, which catalyzes the final step in the lysine biosynthetic pathway in which meso-diaminopimelate is converted into lysine. The *lysA* gene is essential for the strain to grow on minimal medium [31,32].

A conditional auxotrophy-based method for removing selection markers was developed by Brans et al. [33]. This method combines the use of *blaI*, a spectinomycin resistance gene, with a conditional lysine-auxotrophic *B. subtilis* strain (BS1541). The *blaI* gene of *B. licheniformis* encodes a cytoplasmic repressor (BlaI) that negatively regulates the expression of β-lactamase (encoded by *blaP*) in the absence of penicillin. The *B. subtilis* *PlysA* promoter was replaced with the *B. licheniformis* *PblaI* promoter to obtain strain BS1541, thus *blaI* can confer lysine auxotrophy to the strain. The *blaI* cassette, containing *blaI* flanked by two DR sequences, was inserted into the genome of strain BS1541 by homologous recombination and selected for with spectinomycin, and the cassette was removed by a single-crossover recombination between the two DRs. This strategy can be used consecutively to further modify the *Bacillus* chromosome.

A lysine-auxotrophic strain combined with *lacI* A similar method was developed by Zhang et al. [34]. A conditionally lysine-auxotrophic *B. subtilis* strain (BS-PS) was generated by substituting the *PlysA* promoter with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *PspaC* promoter. A vector containing *lacI*, which encodes a repressor of the *PspaC* promoter, and a chloramphenicol resistance gene was used to generate specific integrating vectors. The integration of these vectors into the BS-PS chromosome, and the excision of *lacI* and the chloramphenicol resistance gene, were both achieved by a single crossover at the homologous arm.

**A method based on a histidine-auxotrophic strain**

*hisF* and *hisI* are the last two genes in the *his* operon of the *B. subtilis* 168 chromosome, and are followed by two genes of unknown function, *yvcA* and *yvcB*. An incomplete histidine biosynthesis operon leads to histidine auxotrophy in *B. subtilis*.

Motejadded et al. [35] developed a system based on two plasmids to obtain marker-free recombinant *B. subtilis* strains. One plasmid consists of the 3′-end of *hisF*, the 5′-end of *hisI*, a spectinomycin resistance gene, and a 1.3 kb fragment containing the 3′-end of *yvcA* and the 5′-end of *yvcB*. The other plasmid contains the same *yvc*-region and the 3′-end of *hisI*, but has a complete *hisI* and lacks the antibiotic marker. Spectinomycin-resistant His-auxotrophic mutants are obtained after integration of the first plasmid into the *B. subtilis* genome by double crossover. Insertion of the second plasmid into this mutant leads to a spectinomycin-sensitive His-prototrophic strain. These plasmids were successfully applied to integrate the lipase gene of *Bacillus thermocatenatus* into a *B. subtilis* glucose-regulated system. Morabbi et al. also used this system to perform markerless integration of *Pmut*-mtlIR-H342D into the chromosome of *B. subtilis* [36].

**Site-specific recombination-based genetic engineering strategies**

Site-specific recombination (SSR) systems use recombinases that catalyze recombination between two site-specific recognition sites, which generates a desired DNA integration, deletion, or inversion. SSR systems that are used in bacterial genome engineering include Cre/loxP from bacteriophage P1 [37], Xis/attP from bacteriophage λ [38], and FLP/FRT [39] from *S. cerevisiae*. The recombination efficiency of SSR systems is much higher than that of native recombination systems, which makes them applicable for undertaking multiple sequential mutations of the same chromosome (Figure 3). Recently, Cre/loxP and Xer/dif systems have been used in *B. subtilis* species.

**The Cre/lox system**

The Cre/lox system is a powerful genetic tool [40] that is widely used in eukaryotic and prokaryotic cells and consists of a Cre recombinase and a pair of *loxP* sites. Cre recombinase catalyzes the reciprocal site-specific recombination between the two *loxP* sites, which does not require any host cofactors or accessory proteins. A pair of modified *lox* sites, *lox71* (L) and *lox66* (R), are usually used [41] to minimize genetic instability, as microbial genomes can contain multiple native *loxP* sites that could be identified by Cre. A double mutant *lox72* remnant site, which has weaker binding affinity for Cre, is obtained following recombination of *lox71* and *lox66*, allowing for repeated mutations in a single genetic background [42,43].

Yan et al. [11] developed a genome engineering procedure for *B. subtilis* by combining the mutant Cre/lox system with a fusion PCR method. After the integration of a fusion PCR product (*lox71*-spc/zeo-*lox66* cassette) into the genome, a thermosensitive vector containing *cre*
is introduced to promote recombination between lox66 and lox71 sites. The PCR fusion product is only about 1.5 kb long, reducing the occurrence of PCR errors, which in turn reduces the probability of introducing errors into the chromosome. Three mutations have been successfully integrated into the same background strain using this method. Chromosomally-encoded tetR in B. subtilis was disrupted by insertion of a lox66-aphIII-lox71 kanamycin resistance cassette, and subsequent marker excision by Cre recombinase led to the assembly of a novel tetR allele [44]. Phosphotransacetylase, encoded by pta, was ablated using the Cre/lox system in a strain of B. subtilis 168 engineered to produce L-malate [45]. Finally, five adjacent genes, nagP, gamP, gamA, nagA, nagB, were also deleted using the Cre/lox strategy and a PCR-based chromosome modification method for production of N-acetylglycosamine in B. subtilis [46].

*Bacillus coagulans* is a promising species for producing bulk chemicals from renewable resources in the industrial field, but there are few genetic tools for its modification. Kovacs et al. described a Cre-lox system that uses two plasmids for targeted gene modification in B. coagulans [47]. pMH77 is an integration vector that carries a lox66-cat-lox71 cassette flanked by restriction sites that can be used for cloning homologous regions. pMH66 is a Cre-recombinase plasmid that is used to promote recombination between the two lox sites. The authors used this technique to develop a LacZ reporter assay for measuring gene transcription and to express heterologous D-lactate dehydrogenase.

Although Cre-mediated recombination and excision of the chromosomal sequence between two lox sites is efficient, it does not occur in all cells. To address this, Wang et al. [48] developed a procedure combining Cre recombination and the hen egg white lysozyme gene (*hewl*) as a counter-selectable marker that eliminates the cells carrying the selection cassette. This procedure is based on the beta protein of lambda phage, a single-stranded annealing protein, and employs a single-stranded PCR product containing a lox71-ble-hewl-PR-lox66 cassette to modify a specific gene. The single-stranded DNA can be protected from exonuclease digestion by beta protein, and can be recombined via beta recombinase-catalyzed annealing at the replication fork [39,49]. The beta protein, regulated by promoter PRM in the lambda cI857 PRM–P_R promoter system on the thermosensitive plasmid pWY121, promotes homologous recombination. The *hewl* gene is placed after promoter P_R, which is effective in B. subtilis, and is precisely regulated by the C1857 repressor protein [50]. The efficiency of in-frame deletion using this method can reach 100%. As hen egg white lysozyme is active against Bacillus species, and its encoding gene is distantly related to Bacillus genes [51], it could also be effective in other Bacillus species.

Later, Enyeart et al. [52] combined retargetable mobile group II introns, or “targetrons”, and the Cre/lox system into a versatile platform known as Genome Editing via Targetrons and Recombinases (GETR). Targetrons can be inserted into desired DNA sites with such high efficiency that the inclusion of a selectable marker is not necessary. The lox sites are delivered to specific genomic loci by introns, which enable genomic manipulations, and the added flexibility of RNA hairpins formed by the lox sites enhances the efficiency. GETR is an efficient bacterial genetic engineering approach with broad host-applicability that can be used to generate insertions, deletions, inversions, and one-step cut-and-paste operations.

**The Xer/dif system**

Chromosome dimers, which are formed during the bacterial life cycle, must be resolved by the bacterial cell machinery for efficient chromosome segregation. The Xer/dif site-specific recombination system used by most bacteria resolves these chromosome dimers into monomers using two tyrosine recombinases to perform the recombination reaction at the dif site, which consists of 28–30 bp. Xer recombinases are represented by XerC.
and XerD in Gram-negative bacteria such as E. coli [53], and by CodV and RipX in B. subtilis and other Gram-positive bacteria [54]. Intramolecular Xer recombination can excise a dif-flanked DNA sequence from a chromosomally inserted cassette.

Bloor et al. [55] developed a simple and effective method for genetic modification in bacterial chromosomes based on the Xer/dif system. The insertion cassette used in this method consists of a selectable marker gene flanked by dif sites and homology arms that are homologous to the genomic target. After the insertion cassette integrates into the corresponding chromosomal region via double-crossover recombination, Xer recombinases can integrate the two dif sites into one site, thus removing the selectable marker gene. This method eliminates the requirement for an exogenous SSR system, as Xer recombinases are naturally present in bacteria. Furthermore, a counter-selectable gene is not necessary, as the frequency of Xer recombination is sufficient to allow detection of recombinant clones without antibiotic selection. It is worth noting that introducing multiple dif sites in close proximity can induce deletions of the intervening gene segments, so this method may not be suitable for modifying multiple adjacent genes. Pohl et al. [56] used the Xer system to generate B. subtilis mutants with precise deletions in ten extracytoplasmic proteases that affect recombinant protein secretion.

Toxin gene-based genetic engineering strategies
Toxin-antitoxin (TA) systems usually include a functional element consisting of a biologically active protein molecule and its corresponding inhibitor. TA loci are divided into three different types, but the major part of this monograph is devoted to describing type II loci because of the large numbers of known evolutionarily-independent type II gene families. mazF and ccdB are two well-described toxin genes in the mazEF and ccdAB type II TA systems, respectively, both of which have been applied in genetic modification systems. The toxin gene cassettes and commonly used genetic engineering strategies based on them are shown in Figure 4.

**mazF as a counter-selection marker**
mazEF is one of the best-characterized TA systems. The MazF toxin of E. coli is an mRNA interferase that cleaves cellular free mRNAs specifically at ACA trinucleotides [57] to block protein synthesis, which inhibits growth. MazF expression in both bacterial and mammalian cells induces programmed cell death [58,59].

Zhang et al. presented a simple method, using E. coli mazF as a counter-selection marker, that can be applied to many Bacillus species without prior genetic modification of the host [60]. This method uses a mazF cassette containing mazF under the control of IPTG-inducible Pspac promoter, a spectinomycin resistance gene, and two flanking DR sequences. After the mazF cassette from the linearized delivery vector is integrated into a target chromosome locus via double-crossover recombination, the mazF cassette is removed by a single-crossover event between the two DR sequences. This method requires cloning and takes approximately 2 weeks. In addition, mazF is regulated by the Pspac promoter, which has a low induction rate and can have leaky expression in the absence of an inducer in B. subtilis [61-63]. Such leaky expression of mazF increases the frequency of spontaneous mazF-resistant mutants, which decreases the likelihood of isolating colonies with the designed mutation.

![Figure 4](https://www.microbialcellfactories.com/content/13/1/63)
Morimoto et al. [64] combined the IPTG-inducible expression system with a high-fidelity fusion PCR method to generate marker-free deletion mutants of *B. subtilis*. In this method, sequences were designed for integration and excision of the *mazF* cassette by double- and single-crossover, respectively. This procedure is quicker than the method developed by Zhang et al. [60]. However, it still has some limitations, including possible DNA mutations introduced by the 4.0-kb PCR fusion fragment, the difficulty of assembling different DNA fragments, and some leakiness.

Yu et al. [65] replaced the IPTG-inducible *spac* expression system with the *xyl* expression system from *Bacillus megaterium*, which has tighter transcriptional regulation and a higher induction rate than the *spac* expression system [62,66]. In this system, *mazF* is placed under the control of the *P_{xyl}* promoter, which is repressed by the xylose-responsive repressor XylR in the absence of xylose. However, the long PCR-fusion fragment-generated mutations and spontaneously-generated *mazF*-resistant mutants should also be taken into consideration when using the above method.

Like its counterpart in *B. megaterium*, the inducible *P_{xyl}* promoter of *B. subtilis* also has strict transcriptional regulation [67] and, in fact, *P_{xyl}* from *B. subtilis* W23 shows tighter regulation than *P_{xyl}* from *B. megaterium* [61,62]. Lin et al. [68] constructed a mini-*mazF* cassette containing *P_{xyl}* from *B. subtilis*, *mazF*, and a zeocin resistance gene. The mini-*mazF* cassette is about 2 kb long, which can somewhat reduce the possibility of PCR-induced mutations. The transformation frequency of this cassette is three-fold higher than the above-mentioned *mazF* cassettes, and the rate of spontaneous *mazF*-resistant mutants is low.

ccdB as a counter-selection marker

ccdAB is often used for positive selection of transformants, primarily in *E. coli* strains. Commercially-available systems (e.g. StabyCloning™ and StabyExpress™, Delphi Genetics SA, Charleroi, Belgium) are based on CcdB toxicity against gyrase and allow one-step selection of transformants, ensuring stable vector plasmid maintenance.

Recently, the plasmid F toxin gene *ccdB* was used as a counter-selection marker to construct markerless mutants of *Vibrio splendidus* [69]. A suicide vector carrying *ccdB* under the control of the arabinose *P_{BAD}* promoter, which can be transferred to any *Vibrio* strain by RP4-based conjugation, was developed. The genetic modification system based on this suicide vector requires a two-step allelic exchange procedure. In the presence of arabinose, the counter-selection provided by the integrated vector enabled efficient markerless gene replacement in both *V. splendidus* and *Vibrio cholerae*.

Although *ccdB* has been used as a positive selection marker for a long time, its use for counter-selection in markerless genetic engineering is limited to a few strains and it has not yet been used in *Bacillus* species. Further analysis of *ccdB* and toxin genes from other TA systems is needed to determine whether they may be widely applicable for counter-selection in other species.

**Thermosensitive plasmid-based genetic engineering strategies**

Zakataeva et al. [70] developed a simple method based on a thermosensitive replication plasmid to introduce markerless mutations into the chromosomes of *B. amyloquefaciens*. In this method, a delivery plasmid is efficiently introduced into cells for gene replacement, and a two-step replacement procedure mediated by single-crossover events is used. The procedure is efficient and fast and no counter-selection marker or special strain is required. Although this method is designed for *B. amyloquefaciens*, it has also been successfully adapted to *B. subtilis*. Using this method, Sheremet et al. constructed a series of markerless *B. amyloquefaciens* strains to produce inosine and 5-aminomimidazole-4-carboxamide ribonucleoside [71].

**Transconjugation-based genetic engineering strategies**

In some applications, *B. licheniformis* and *B. megaterium* outperform the better-studied microbial model, *B. subtilis*. However, commonly-used methods for genetic modification of these strains, such as protoplast transformation, are time-consuming and complicated. Recently, some easy markerless deletion methods based on transconjugation have been developed.

**A sacB-based transconjugation system for *B. megaterium***

*B. megaterium* is an industrially-important species, as it has been used to produce heterologous proteins and valuable enzymes. However, genetic manipulation of *B. megaterium* is difficult, primarily because of low transformation efficiency. Richhardt et al. [72] developed a simple and efficient transconjugation method for *B. megaterium*, combining a known transconjugation method [73] and *B. subtilis* sacB, which encodes levansucrase. The activity of this enzyme in the presence of sucrose is lethal to *E. coli* [74], so it is used as a counter-selection marker to eliminate the *E. coli* donor cells after mating. The transfer efficiency of this method is approximately $5 \times 10^{-5}$ transconjugants/recipient, which is sufficient to allow direct selection of mutants in a one-step procedure.

**A transconjugation plasmid-system in *B. licheniformis***

Rachinger et al. [75] established a markerless gene modification method for *Bacilli* species without natural competence, such as *B. licheniformis*. Chromosomal gene deletion is accomplished by the pKVM series of conjuga
tive shuttle vectors, which contain regions flanking the
target gene. These shuttle vectors carry the temperatureresultsensitive origin of replication from pE194ts and a thermostable β-galactosidase, allowing blue/white screening of recombinant clones on X-gal-containing agar plates, and can be conjugated to B. licheniformis and B. subtilis strains. Integration of the vector at the target locus, and its subsequent excision, are both mediated by homologous recombination and identified based on appropriate selection markers. These pKVM vectors can be used to efficiently generate deletions and insertions in B. licheniformis and other Bacillus strains.

Proposed methods for genetic engineering of Bacillus species

Tetracycline-dependent conditional gene knockout in B. subtilis

The tetracycline repressor (TetR) and its reverse mutant (revTetR) can be used for reversible, tetracycline-dependent induction and silencing of gene expression, respectively. Kamionka et al. [76] used both of these approaches in B. subtilis, as an example of a Gram-positive bacteria. In this system, the genomic spoVG-lacZ fusion gene is regulated by one or two tet operators, and either TetR or revTetR is controlled by different promoters, allowing precise adjustment of regulatory windows. TetR or revTetR turn expression on or off, respectively, when anhydrotetracycline is added, which means these two components can be used to construct conditional knockouts in B. subtilis and many other Gram-positive bacteria.

oroP from Lactococcus lactis as a counter-selection marker

The orotate transporter of L. lactis, encoded by oroP, mediates 5-fluoroorotate sensitivity in B. subtilis 168, E. coli XL1-Blue, and 5-fluoroorotate-sensitive lactococci. oroP is necessary for pyrimidine-auxotrophic derivative strains to use orotate as a sole pyrimidine source [77].

Solem et al. [78] developed a selection/counter-selection vector, pCS1966, which harbors oroP and can only replicate in E. coli. This plasmid can be used for homologous recombination at a specific site, and for integration at bacteriophage attachment sites. The plasmid contains an erythromycin-resistance gene for positive selection of cassette integration, and orotate utilization can be used for counter-selection and cassette excision in a pyrimidine auxotrophic mutant. As oroP can be functionally expressed in B. subtilis, its use for counter-selection can potentially be exploited in this species.

bgl/lacZ as counter-selection markers

β-glucosidase, encoded by bgl, can cleave 5-bromo-4-chloro-3-indolyl (BCI) to produce an indoxyl derivative that is toxic to bacteria. Angelov et al. [79] described a markerless mutation method that uses bgl and lacZ as counter-selection markers, and demonstrated the method in the thermophile Thermus thermophilus HB27 and in Micrococcus luteus ATCC 27141. This method uses a delivery plasmid containing the counter-selection markers and flanking regions of the target gene for efficient gene replacement in a two-step replacement process mediated by single crossover events. As Bacillus species are also sensitive to BCI substrate cleavage, this approach could be used to generate markerless chromosomal mutations in Bacillus.

Strategy comparison and prospects

Genome engineering strategies usually require two steps: the integration of a disruption cassette into the genome, and the excision of a selectable marker. Examples of these gene modification strategies, classified based on the different procedures used, are shown in Figure 5. Plasmid-borne disruption cassettes can integrate into the genome by single- or double-crossover, whereas those in PCR fragments usually integrate via the latter mechanism.
Restriction endonuclease/ligase-dependent methods are not compatible with large scale approaches, and it generally takes about 2 weeks to complete a marker-free modification. In these methods, single-crossover events have a higher rate of positive recombinants in the cassette integration step than in double-crossover events, although the number of false-positive recombinants will increase in the selection marker excision step. In comparison, methods that use fusion PCR or long-flanking homology PCR techniques to generate the disruption cassette can modify a target gene more rapidly; however, these methods are prone to point mutations and may have difficulties in assembling different DNA fragments. High-fidelity PCR would reduce the incidence of point mutations in short fragments (<4 kb) [80].

Marker excision is usually mediated by a single-crossover event, and the efficiency of this step largely determines the overall success of the genetic modification process. SSR can be used to efficiently eliminate the selection marker from the mutated locus, but it leaves remnant sequences (scars) at the targeted site. Some methods using counter-selection markers are scarless, but they are less efficient than methods based on SSR. Thus, methods combining SSR with counter-selection markers have emerged.

Many methods, such as those derived from app deletion, can only be used in strains that have a specific gene mutated, which limits their application. Hence, methods that require no such prior modifications in the host and can be applied to some Bacillus species to satisfy the strong demand for a universal unmarked delivery system will receive much attention.

In general, an innovative method to modify genomes of Bacillus species should have certain characteristics: 1) it allows markerless or scarless genome manipulation that is both efficient and precise; 2) it has no requirement for any prior mutation prior to genetic modification; 3) it can be used for system-level genetic modifications or can generate multiple genomic mutations simultaneously.

Based on the above analysis, efforts to improve genetic modification technology for Bacillus species should focus on: 1) optimizing existing homologous-recombination-based genetic modification methods; 2) introducing more advanced technologies from other species into Bacillus species; 3) developing new combinatorial engineering tools for genome-wide modification, such as global transcriptional engineering [81], tractable multiplex recombineering [82], and multiplex automated genome engineering [83]; 4) adapting novel genome editing engineering technology, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated sequences and similar multi-loci editing systems, to Bacillus species [84,85].

Conclusions

Bacillus species have become important platforms for producing various enzymes and chemicals. A vast range of cellular phenotypes can be obtained in Bacillus species by regulating and modifying the corresponding metabolic pathways at global and gene-specific levels. Advances in genetic engineering strategies have helped realize the potential of Bacillus species as production hosts for manufacturing commodities, and make Bacillus species competitive with the traditional industrial microbes E. coli and S. cerevisiae.

Many useful tools for genetic modification of Bacillus species have been developed in recent years. In this review, we summarized and compared the design principles of current genetic engineering strategies and their recent progress. These strategies still have their own challenges and limitations, so comprehensive and efficient tools for systems-level genetic modifications are still required. We also detailed future research prospects for developing novel genetic modification systems for Bacillus species, which are expected to inspire further interest and advance studies in related fields.

Abbreviations

UPRTase: Uracil-phosphoribosyltransferase; 5-FU: 5-Fluorouracil; DR: Direct repeats; MS: Master strain; DSB: Double-strand break; OPRTase: Orotate phosphoribosyltransferase; OMPdecase: Orotidine 5′-phosphate decarboxylase; IPTG: Isopropyl-β-D-thiogalactopyranoside; SSR: Site-specific recombination; GETR: Genome Editing via Targetrons and Recombinases; TA: Toxin-antitoxin; TetR: Tetracycline repressor; BCI: 5-bromo-4-chloro-3-indolyl; CRISPR: Clustered regularly interspaced short palindromic repeats.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

All authors defined the topic of the review and wrote, read and approved the manuscript. Both authors read and approved the final manuscript.

Acknowledgements

The authors would like to express their thanks to financial supports from State Key Development Program for Basic Research of China (973 Program, 2013CB733600), National Nature Science Foundation of China (31200036, 31370089), the Key Projects in the Tianjin Science & Technology Pillar Program (12JCZDSY12700, 11JCZDSY08500, 11JCZDSY08400). The authors gratefully acknowledge the support of K. C. Wong Education Foundation, Hong Kong.

Author details

1Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China. 2Key Laboratory of Systems Microbial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China. 3National Engineering Laboratory for Industrial Enzymes, Tianjin 300308, China.

Received: 3 January 2014 Accepted: 28 April 2014

Published: 3 May 2014

References

1. Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phun TH, Pfeifer B, Stephanopoulos G: Isoprenoid pathway optimization for Taxol precursor overproduction in Escherichia coli. Science 2010, 330(6000):70–74.
2. De Jong B, Sewers V, Nielsen J: Systems biology of yeast: enabling technology for development of cell factories for production of advanced biofuels. Curr Opin Biotechnol 2012, 23(4):624–630.

3. Schumann W: Production of recombinant proteins in Bacillus subtilis. Adv Appl Microbiol 2007, 62:137–189.

4. Schallmey M, Singh A, Ward OP: Developments in the use of Bacillus species for industrial production. Can J Microbiol 2004, 50:111–17.

5. Perkins JW, Sauer U, Hohmann HP: Metabolic engineering of B. subtilis. In Metabolic pathway engineering handbook. Edited by Smolke CD, Nielsen J. Texas: CRC press; 2009.

6. Liu L, Liu Y, Shin H-D, Chen RR, Wang NS, Li J, Du G, Chen J: Developing Bacillus spp. as a cell factory for production of microbial enzymes and industrially important biochemicals in the context of systems and synthetic biology. Appl Microbiol Biotechnol 2013, 97(14):6113–6120.

7. Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, Bertero MG, Bessieres P, Bolotin A, Borchart S, Boursier R, Boursin L, Brans A, Braun M, Brignell SC, Brion S, Bruschi CV, Caldwell B, Capuano V, Carter NM, Choi SK, Codani J, Connerton IF, Cummings NJ, Daniel RA, Denizot F, Devine KM, Düsterhöft A, Ehrlich SD, et al: The complete genome sequence of the gram-positive bacterium Bacillus subtilis. Nature 1997, 390(6657):249–256.

8. Chen XH, Koumoutsi A, Eisenreich A, Schneider K, Heinemeyer I, Morgenstern B, Voss B, Hess WR, Reva O, Junge H, Voigt B, Jungblut PR, Aum, Umer S, Bäumer S, Henne A, Liesegang H, Merkl R, Ehrenreich A, Ogasawara N: Enhanced recombinant protein productivity by genome reduction in Bacillus subtilis. DNA Res 2008, 15(2):73–81.

9. Tanaka K, Henry CS, Zimmer JF, Jolivet E, Cohoon MP, Xia F, Bidnerko V, Ehrlich SD, Stevens RL, Noroit P: Building the reperoire of dispensable chromosome regions in Bacillus subtilis entails major refinement of cognate large-scale metabolic model. Nucleic Acids Res 2012, 41(1):687–699.

10. Shi T, Wang G, Wang Z, Fu J, Chen T, Zhao X: Establishment of a markerless mutation delivery system in Bacillus subtilis stimulated by a double-strand break in the chromosome. PLoS ONE 2013, 8(11):e81370.

11. Wernhoff S, Meinhard F: Generation of biologically contained, readily transformable, and genetically manageable mutants of the biotechnologically important Bacillus pumilus. Appl Microbiol Biotechnol 2013, 97(7):7805–7819.

12. Suzuki H, Murakami A, Yoshida K: Counterselection system for Geobacillus kaustophilus HTA426 through disruption of pyrF and pyrR. Appl Environ Microbiol 2012, 78(20):7376–7383.

13. Kobayashi K, Ehrlich SD, Albertini A, Arami G, Andersen KB, Arnaud M, Asai K, Ashikaga S, Aysmerich S, Besierers P, Boland F, Brignell SC, Brion S, Bunai K, Chapsis J, Christiansen LC, Danchin A, Dabarbourille M, Dervyn E, Deuringer E, Devine K, Devine SK, Deosen O, Errington J, Fillinger S, Foster SJ, Fujita Y, Galbazi A, Gardan R, Arechevis C: Essential Bacillus subtilis genotypes. Proc Natl Acad Sci U S A 2003, 100(8):4678–4683.

14. Rosner A: Control of Lysis biosynthesis in Bacillus subtilis: Inhibition of dianiminopeptidase deacylation by Lysine. J Bacteriol 1975, 121(10):20–28.

15. Brans A, Filee P, Chevigne A, Claessens A, Joris B: New integrative vector to generate Bacillus subtilis recombinant strains free of selectable markers. Appl Environ Microbiol 2004, 70(12):2411–2417.

16. Zhang C, Zhang X, Yao Z, Lu Y, Lu F, Lu Z: A new method for multiple gene inactivations in Bacillus subtilis168, producing a strain free of selectable markers. Can J Microbiol 2011, 57(5):437–436.

17. Morabbi Heravi K, Werznel M, Altenbuchner J: Regulation of ntl operon promoter of Bacillus subtilis: requirements of its use in expression vectors. Microb Cell Fact 2011, 10:113.

18. Le Borgne S, Bolivar F, Gosset G: Plasmid vectors for marker-free chromosomal insertion of genetic material in Escherichia coli. Methods Mol Biol 2004, 267:135–143.

19. Zúbik E, Scutt C, Meyer P: Intrachromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes. Nat Biotechnol 2000, 18(4):442–445.

20. Datenson KA, Wanner BL: One-step inactivation of chromosomal genes in Escherichia coli K–12 using PCR products. Proc Natl Acad Sci U S A 2000, 97(12):6640–6645.

21. Kuhn R, Torres RM: Cre/loxP recombination system and gene targeting. Methods Mol Biol 2002, 180:175–204.

22. Albert H, Dale EC, Lee E, Ow CW: Site-specific integration of DNA into wild-type and markerless loci sites placed in the plant genome. Plant J 1995, 7(4):649–659.

23. Lambert JM, Bongers RS, Klerebezem M: Cre-lox-based system for multiple gene deletions and selectable-marker removal in Lactobacillus plantarum. Appl Environ Microbiol 2006, 73(4):1126–1135.

24. Suzuki N, Nomura H, Tsuge Y, Inui M, Yukanoue H: New multiple-deletion method for the Corynebacteriaceae glutamimycin genome, using a mutant lox sequence. Appl Environ Microbiol 2005, 71(12):4842–4840.

25. Bentam R, Kolb M, Hilten W: In vivo Activation of Tetracycline Repressor by Cre/lox-Mediated Gene Assembly. J Microbiol Biotechnol 2009, 19(3):136–145.

26. Lu M, Wen J: Engineered Bacillus subtilis 168 produces L-malate by heterologous biosynthesis pathway construction and lactate dehydrogenase deletion. World J Microb Biotechnol 2012, 29(1):33–41.
Antimicrobial peptides derived from hen egg lysozyme with inhibitory effect against Bacillus subtilis. Food Control 2007, 18(2):173–178.

Pohl S, Bhavsar G, Hulme J, Bloor AE, Misri S, Leckebony MW, Radford DS, Smith W, Wipat A, Williamson ED, Harwood CR, Canenburgh RM. Proteomic analysis of Bacillus subtilis strains engineered for improved production of heterologous proteins. Proteomics 2013, 13(22):3298–3308.

Yang Y, Zhang J, Hara H, Kato I, Inouye M. mazF as a counter-selectable marker for unmarked chromosomal manipulation in Bacillus subtilis. Nucleic Acids Res 2006, 34(9):e71.

Bhavsar AP, Zhao X, Brown ED. Development and characterization of a xylose-dependent system for expression of cloned genes in Bacillus subtilis: conditional complementation of a teichoic acid mutant. Appl Environ Microbiol 2001, 67(1):403–410.

Hart B, Weihl W, Wiegert T, Homuth G, Schumann W. Development of a new integration site within the Bacillus subtilis chromosome and construction of compatible expression cassettes. J Bacteriol 2001, 183(8):2696–2699.

Vagner V, Dervyn E, Ehrlich SD. A vector for systematic gene inactivation in Bacillus subtilis. Microbiology 1998, 144:3097–3104.

Morimoto T, Aka K, Ozaki K, Ogawaara N. A new simple method to introduce marker-free deletions in the Bacillus subtilis genome. Gene Genet Syst 2009, 84(1):315–318.

Yu H. Efficient and precise construction of markerless manipulations in the Bacillus subtilis genome. J Microbiol Biotechnol 2010, 20(1):45–53.

Kim I, Mogk A, Schumann W. A xylose-inducible Bacillus subtilis integration vector and its application. Gene 1996, 181:71–76.

Lewis PJ, Marston AL. GFP vectors for controlled expression and dual labelling of protein fusions in Bacillus subtilis. Gene 1999, 227(1):101–110.

Lin Z, Deng B, Liao Z, Wu B, Xu X, Li D, Zhang R. A versatile mini-mazF-cassette for marker-free targeted genetic modification in Bacillus subtilis. J Microbiol Methods 2013, 95(2):207–214.

Le Roux F, Binesse J, Saulnier D, Mazel D. Construction of a vibrio splendidus mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. Appl Environ Microbiol 2006, 73(3):777–784.

Zakateva NP, Nikitina OV, Gronsky SV, Romanenkov DV, Livichts VA. A simple method to introduce marker-free genetic modifications into the chromosome of naturally nontransformable Bacillus amyloliquefaciens strains. Appl Microbiol Biotechnol 2009, 85(4):1201–1209.

Sheremet AS, Gronsky SV, Akhmadzyan RA, Novskova AE, Livichts VA, Shakulov RS, Zakateva NP. Enhancement of extracellular purine nucleoside accumulation by Bacillus strains through genetic modifications of genes involved in nucleoside export. J Ind Microbiol Biotechnol 2010, 38(1):85–90.

Richardt J, Lensen M, Meinhardt F. An improved transconjugation protocol for Bacillus megaterium facilitating a direct genetic knockout. Appl Microbiol Biotechnol 2010, 86(6):1959–1965.

Muro A MA, Priest FG. Construction of chromosomal integrants of Bacillus subtilis 2362 by conjugation with Escherichia coli. Res Microbiol 2000, 151:547–555.

Gay P, Cacq DL, Steinmetz M, Verkleym T, Kado CI. Positive selection procedure for entrapment of insertion sequence elements in Gram-negative bacteria. J Bacteriol 1985, 164:918–921.

Rochinger M, Bauch M, Strittmatter A, Bongaerts J, Evers S, Maurer KH, Daniel R, Liebl W, Liesegang E, Ehrenreich A. Size unlimited markerless deletions by a transconjugative plasmid-system in Bacillus licheniformis. J Biotechnol 2013, 167(4):365–369.

Karnionka A, Bertram R, Hillen W. Tetracycline-dependent conditional genetic knockout in Bacillus subtilis. Appl Environ Microbiol 2005, 71(2):728–733.

Defeo E, Kyger MB, Martinussen J. The orotate transporter encoded by orOP from Lactococcus lactis is required for orotate utilization and has utility as a food-grade selectable marker. Microbiology 2007, 153(11):3645–3659.

Solem C, Defeo E, Jensen PR, Martinussen J. Plasmid pCS1956, a new selection/counterselection tool for lactic acid bacteria strain construction based on the orOP gene, encoding an orotate transporter from Lactococcus lactis. Appl Environ Microbiol 2008, 74(15):4772–4775.

Angelov A, Li H, Geissler A, Leis B, Liebl W. Toxicity of inositol derivative accumulation in bacteria and its use as a new counterselection principle. Syst Appl Microbiol 2013, 36(8):585–592.

Shenchuk NA, Brykojn AV, Nusinovich YA, Cabello FC, Sutherland M, Ladisch S. Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. Nucleic Acids Res 2004, 32(21):e19.

Alper H, Stephanopoulos G. Global transcription machinery engineering: a new approach for improving cellular phenotype. Metab Eng 2007, 9(3):258–267.

Warner JR, Reeder PJ, Kaimpouri-Fard A, Woodruff LB, Gill RT. Rapid profiling of a microbial genome using mixtures of barcoded oligonucleotides. Nat Biotechnol 2010, 28(8):856–862.

Wang H, Isacs FJ, Carr PA, Sun ZZ, Xu G, Forest CR, Church GM. Programming cells by multiplex genome engineering and accelerated evolution. Nature 2009, 460(7257):894–898.

Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 2013, 31(3):233–239.

Chapentier E, Doudna JA. Biotechnology: Rewriting a genome. Nature 2013, 495(7439):50–51.

http://www.microbialcellfactories.com/content/13/1/63

Dong and Zhang Microbial Cell Factories 2014, 13:63

doi:10.1186/1475-2859-13-63

Cite this article as: Dong and Zhang: Current development in genetic engineering strategies of Bacillus species. Microbial Cell Factories 2014 13:63.