Microbial Cell Factories

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BsuMI regulates DNA transformation in *Bacillus subtilis* besides the defense system and the constructed strain with BsuMI-absence is applicable as a universal transformation platform for wild-type *Bacillus*

Zhao Xingya[1,2,3], Fu Xiaoping[1,3,4], Zhen Jie[1,2,4], Yang Jun[1,4], Zheng Hongchen[1,2,3,4*], Bai Wenqin[1,3,4*] and Song Hui[1,2,3,4]

Abstract

Background To effectively introduce plasmids into *Bacillus* species and conduct genetic manipulations in *Bacillus* chassis strains, it is essential to optimize transformation methods. These methods aim to extend the period of competence and enhance the permeability of the cell membrane to facilitate the entry of exogenous DNA. Although various strategies have been explored, few studies have delved into identifying metabolites and pathways associated with enhanced competence. Additionally, derivative *Bacillus* strains with non-functional restriction-modification systems have demonstrated superior efficiency in transforming exogenous DNA, lacking more explorations in the regulation conducted by the restriction-modification system to transformation process.

Results Transcriptomic comparisons were performed to discover the competence forming mechanism and the regulation pathway conducted by the BsuMI methylation modification group in *Bacillus. subtilis* 168 under the Spizizen transformation condition, which were speculated to be the preferential selection of carbon sources by the cells and the preference for specific metabolic pathway when utilizing the carbon source. The cells were found to utilize the glycolysis pathway to exploit environmental glucose while reducing the demand for other phosphorylated precursors in this pathway. The weakening of these ATP-substrate competitive metabolic pathways allowed more ATP substrates to be distributed into the auto-phosphorylation of the signal transduction factor ComP during competence formation, thereby increasing the expression level of the key regulatory protein ComK. The expression of ComK upregulated the expression of the negative regulator SacX of starch and sucrose in host cells, reinforcing the preference for glucose as the primary carbon source. The methylation modification group of the primary protein BsuMI in the restriction-modification system was associated with the functional modification of key enzymes in the oxidative phosphorylation pathway. The absence of the BsuMI methylation modification group resulted in a decrease

*Correspondence:
Zheng Hongchen
zheng_hc@tib.cas.cn
Bai Wenqin
baiwq@tib.cas.cn

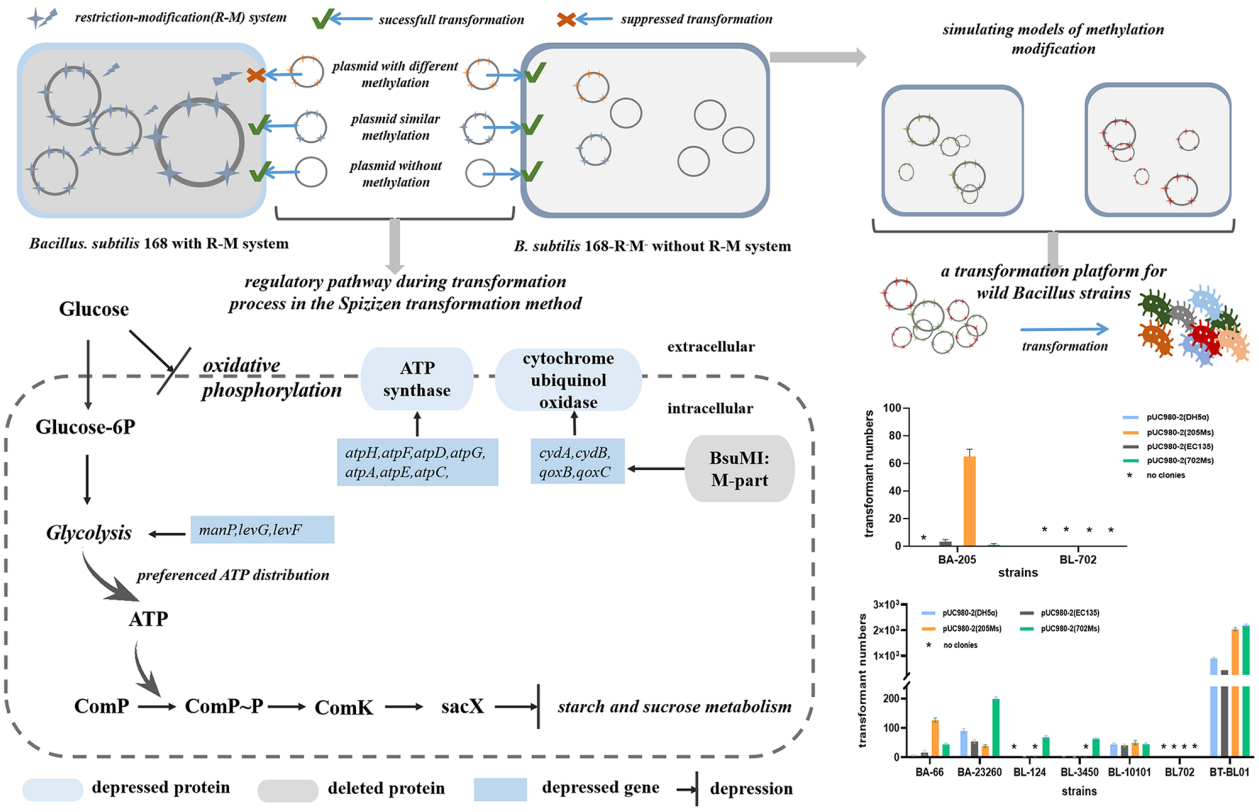
Full list of author information is available at the end of the article

in the expression of subunits of cytochrome oxidase, leading to a weakening of the oxidative phosphorylation pathway, which promoted the glycolytic rate of cells and subsequently improved the distribution of ATP molecules into competence formation. A genetic transformation platform for wild-type *Bacillus* strains was successfully established based on the constructed strain *B. subtilis* 168-R$^-$M$^-$ without its native restriction-modification system. With this platform, high plasmids transformation efficiencies were achieved with a remarkable 63-fold improvement compared to the control group and an increased universality in *Bacillus* species was also obtained.

Conclusions The enhanced competence formation mechanism and the regulation pathway conducted by the functional protein BsuMI of the restriction-modification system were concluded, providing a reference for further investigation. An effective transformation platform was established to overcome the obstacles in DNA transformations in wild-type *Bacillus* strains.

Keywords Plasmid transformation, Restriction-modification system, Regulatory mechanism, *Bacillus* species

Graphical Abstract



Background

Bacillus, as a host cell generally recognized as safe, has been one of the optimal chassis cells in synthetic biology for its easy incubation, short growth cycle, high fermentation density, and unique advantages in efficient production of recombinant proteins [1–3]. However, many *Bacillus* strains cannot be genetically manipulated for domestication, especially some wild types, due to a lack of effective transformation methods [4–6]. Therefore, effective transformation and genetic manipulation methods are being developed as one of the main research focuses in *Bacillus* species, providing technical support for the development and application of *Bacillus* chassis cells.

According to previous studies, the transformation of *B. subtilis* can be divided into two processes of competence forming and DNA adsorption system, as briefly shown in Fig. 1A and B as a summary of previous elucidations of DNA transformations in *B. subtilis* [7–9]. Cells are regulated to the competence state by a complex and extensive signal transduction system under the stimulation of environmental factors such as nutrient deprivation and increased cell density [10, 11]. Competence cells then complete the adsorption, uptake, and recombination of exogenous DNA through a series of regulatory events

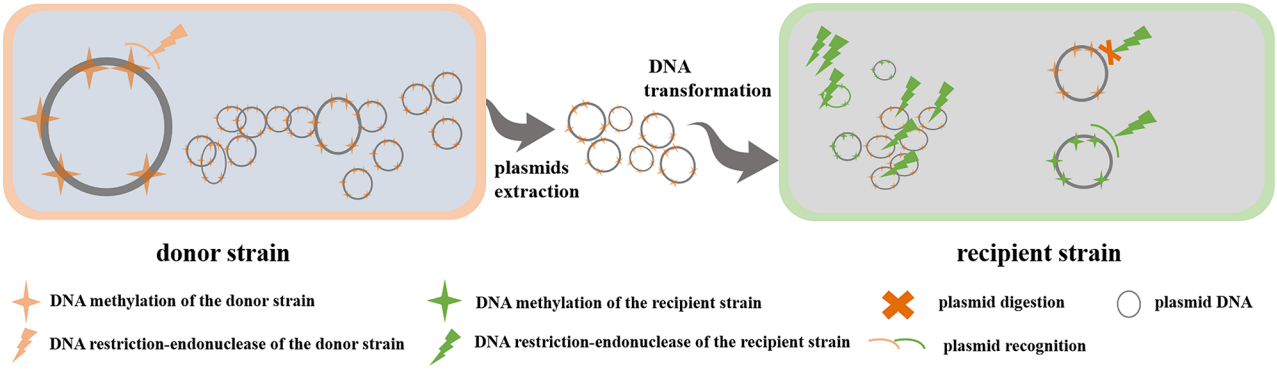


Fig. 1 A diagrammatic sketch of plasmids transformation in different *Bacillus* strains

[12]. The transcriptional regulator ComK is essential for the expressions of a series of genes related to DNA uptake and DNA recombination specific in competent cells [13]. Based on these progressive investigations in competence formation procedure, researchers have carried out strategies on accelerating the competence formation, prolonging the presentation period of competence state and breaking the cell membrane to promote the transformation efficiency of exogenous DNA into the host cell [14–17].

The Spizizen transformation, as one of the most classical method, is still frequently used with good efficiencies [18]. Although many transformation methods have been developed by optimizing the conditions, rare studies have been focused on the metabolic regulation to investigate the reason for enhanced transformation under the Spizizen transformation condition [14, 19]. As the researches went further and deeper, it became more and more clear that the DNA transformation was not only related to the cell state, but also influenced by the restriction-modification system in host cells [20–22]. R-M systems defend against invaders, such as phages and plasmids by eliminating them to protect host cells, as shown in Fig. 1C [23]. For example, SP10 phages extracted from the wild-type strain and the strongly methyl-modified strain were found to be resistant to digestion by the restriction-endonuclease in the host strain, while those treated with *B. subtilis* BSU54 (m^-r^-) with a deficient R-M system could not be transferred [24]. Some derivative strains with non-functional R-M systems have also been used for transformations [25, 26]. However, the pathways of the native restriction modification system involved to influence plasmid transformation have rarely been studied [27]. In our work, transcriptomic comparisons were performed to discover the competence forming mechanism and the regulation pathway conducted by the BsuMI methylation modification group in the Spizizen transformation method.

Considering the importance of the R-M system during plasmid transformation in *Bacillus*, Zhang et al. constructed a strain named *Escherichia coli* EC135 with expressed methyltransferases selected from *Bacillus* strains, facilitating plasmids transformations in *Bacillus amyloliquefaciens* TA208 and *Bacillus cereus* ATCC 10,987 [28]. But the wide use of the strain EC135 is limited as most expression vectors in *Bacillus* cannot shuttle between *E. coli* and *Bacillus.* In this work, inspired by the construction of EC135, we constructed a strain with an inefficient R-M system to mimic DNA-methylation modifications and achieve efficient transformations of non-shuttle plasmids in wild-type *Bacillus* strains.

Results

Construction of B. subtilis strains with defective R-M systems and plasmid transformation in constructed strains

B. subtilis 168 was selected as the initial strain due to its wide-spread use as a model strain and its ease of genetic manipulation. Its restriction-modification system (R-M system) was analyzed on the REBASE database and shown in Fig. 2A. BsuMI obtains both functions of restriction endonuclease (REase) and methyltransferase (MTase) with coding genes *bsuRA-bsuRB-bsuRC* and *bsuMA-bsuMB*, with a specific DNA sequence "CTCGAG", the same recognition sequence as the DNA restriction endonuclease XhoI. These gene clusters were sequentially knocked out using the CRISPR/Cas9 double plasmids system (Fig. S1B). The constructed strains were named as *B. subtilis* 168-R^- and 168-R^-M^-, which were referred as 168-R^- and 168-R^-M^- in the following part. The growth curves of these constructed strains in LB medium were examined and presented in Fig. 2B, with slower growth in the early stage of growth, but caught up in the late stage of logarithmic time. Meanwhile, both strains 168-R^- and 168-R^-M^- grew better than 168 in both GM1 and GM2 medium of the Spizizen transformation with superior cell density and duration time of the exponential growth state than 168 (Fig. 2C).

A high copy number plasmid pUC980-2 constructed in our previous work and the commonly used plasmid pHT43 were hired to transfer the constructed strains

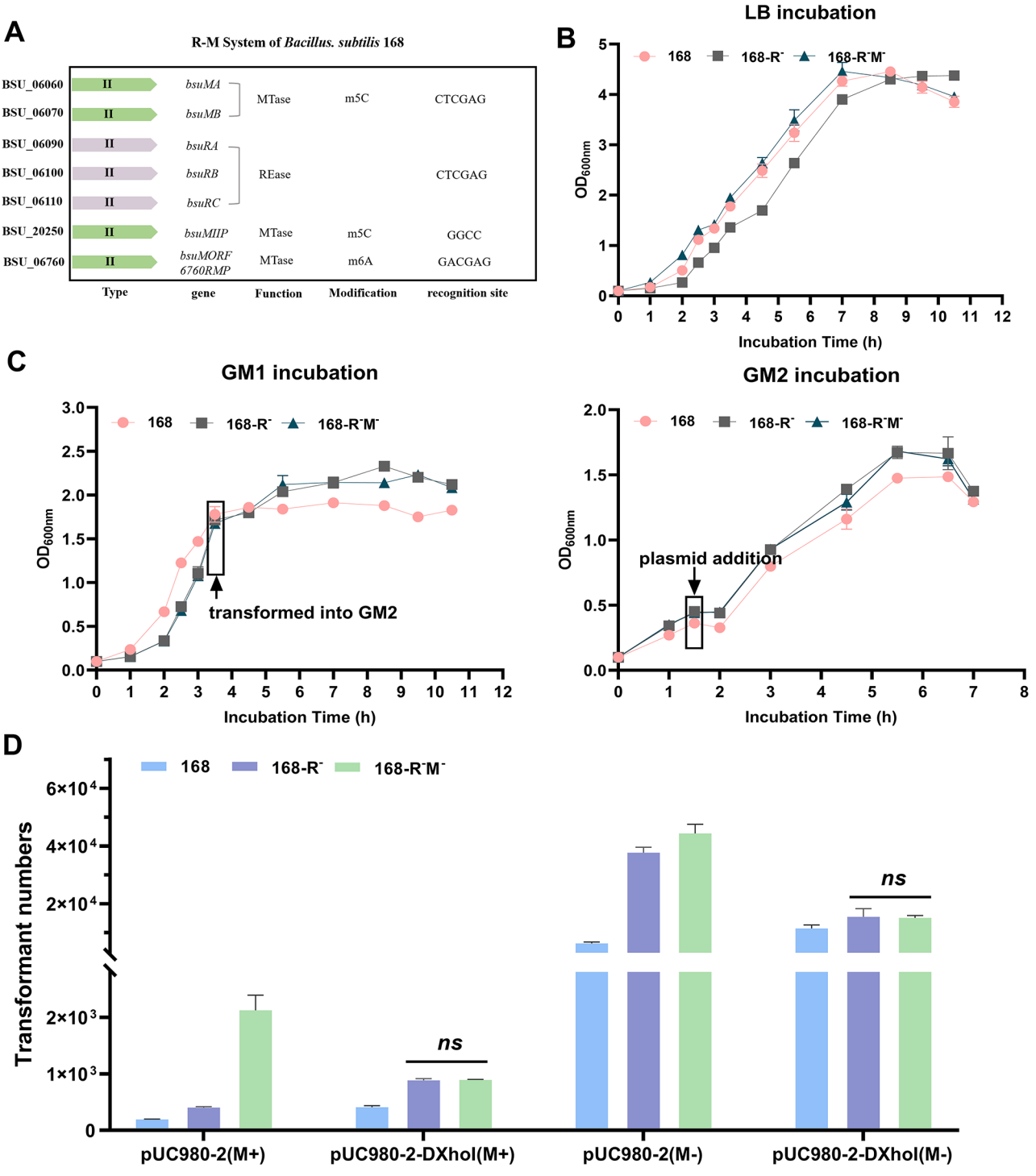


Fig. 2 Growth curves and transformation efficiencies of *B. subtilis* strains with inefficient R-M systems. The R-M system in *B. subtilis* 168 was analyzed and strains with inefficient R-M system were constructed based on these results. These strains were further determined for growth curves and plasmids transformations. **A** the analysis result of the R-M system in *B. subtilis* 168. **B** the growth curves of strains in LB medium. **C** the growth curves of GM1- and GM2-incubations in the Spizizen transformation. **D** transformations of plasmid pUC980-2 in constructed strains. Plasmids modifications: plasmids extracted from *E. coli* DH5α were modified with its native methylation system and considered as group "M+". The recognition sequence "CTCGAG" (XhoI recognition site) of the native 168 R-M system was deleted from pUC980-2 and pHT43, resulting in plasmids pUC980-2-DXhoI and pHT43-DXhoI. In addition, unmethylated plasmids extracted from *E. coli* 110 were grouped as "M-"and compared with the normally methylated (M+) plasmids. All experiments were conducted with three biological replicates. $p < 0.05$ in all groups, except for not-significant results in the pUC980-2-DXhoI(M+) and pUC980-2-DXhoI(M-) transformations between the strains 168-R⁻ and 168-R⁻M⁻. ***ns*** not significant

with different modifications. The prevention of the R-M system to the DNA access process was strongly demonstrated by the increasing transformation efficiencies in strain 168 along with the decreasing modification on the plasmid DNA and successively increased transformation efficiencies in 168-R$^-$ (Fig. 2D). Improvements in transformation efficiencies were also observed when the BsuMI methylation subunit BsuMA-BsuMB was deleted with the highest transformant number of $4.4\times10^4(\pm3215)$ in 168-R$^-$M$^-$, indicating that the BsuMI methylation subunit also exerted a blocking effect on the transformation process. However, this blocking effect was attenuated with the XhoI-deletions in plasmids pUC980-2-DXhoI(M+) and pUC980-2-DXhoI(M-). Similar trends were also observed for pHT43 transformations in Figure S2. A certain curiosity about "whether and how the BsuMI methylation subunit regulates the Spizizen transformation process was stimulated. Unexpectedly, some of these results were contrary to the expectation that the transformation efficiency increased along with the reduced modifications and recognition sites on plasmid pUC980-2. These opposite results were not observed in pHT43 transformations, suggesting that the regulatory mechanism might be dependent on the plasmid replication type and deserves more investigations.

Transcriptomic analysis of differential genes in strains during the spizizen transformation

After excluding outlier samples, the correlation among the remaining samples was above reliable. The key regulatory gene *comK* involved in competence formation in *Bacillus subtilis* and the key gene cluster *bsuRA* (shown as *ydiR*), *bsuRB*, *bsuRC*, *bsuMA*, and *bsuMB* of the restriction-modification system were selected as target genes for validation of the transcriptome sequencing results. As shown in the Supplementary Figure S3A, the cluster heatmap confirmed that the corresponding key genes of the restriction-modification system were not expressed in strains 168-R$^-$ and 168-R$^-$M$^-$. Additionally, under the Spizizen cultivation condition, the expression level of the host strain's *comK* gene was higher compared to conventional LB cultivation conditions. The validation results also reflected the reliability of the sequencing data. Prefix in the sample name stood for different incubation conditions: "L" meant LB incubation; "S" meant the Spizizen transformation condition.

First, the differential genes of the three strains in the Spizizen medium were analyzed, as shown in Fig. 3A and Figure S3B. Among the differential genes between the recombinant strains 168-R$^-$ and 168-R$^-$M$^-$ and the initial strain 168, there were 10 common genes involved in pathways related to cell growth such as glycolysis, carbon metabolism, and biosynthesis of amino acids (Fig. S3D). Due to the small number of genes, more focused analysis could not be conducted. The enrichment results of the differential genes unique to these two comparison groups were not significant.

Then we focused on the 132 independent differential genes between the two strains 168-R$^-$ and 168-R$^-$M$^-$, which had partial and complete deletions of the restriction-modification system, respectively. KEGG enrichment analysis showed that these genes were mainly concentrated in the oxidative phosphorylation pathway (GeneRatio: 13/58) (Fig. 3B). The specific expression fold changes of these 13 genes are shown in Fig. 3C. Compared with the initial strain 168, the expression levels of these genes were reduced in the strain 168-R$^-$M$^-$, which had a further deletion of the BsuMI methylation subunit, while there was no down-regulation trend in the expression levels of these genes in the strain 168-R$^-$, which only had the BsuMI restriction group deleted but retained the methylation subunit. The summary of gene functions was shown in Table 1. This result indicated that the deletion of the BsuMI methylation group inhibited the oxidative phosphorylation pathway in *Bacillus subtilis* 168. To determine whether this inhibitory effect is only manifested when the cells are in a specific state, we conducted transcriptome analysis on cells under two different cultivation conditions.

Transcriptomic comparisons of each strain under the spizizen transformation and LB cultivation

Compared to growth in LB medium, under the Spizizen cultivation conditions, strain 168 had 980 up-regulated genes and 1175 down-regulated genes, strain 168-R$^-$ had 943 up-regulated genes and 1160 down-regulated genes, and strain 168-R$^-$M$^-$ had 851 up-regulated genes and 1177 down-regulated genes (Supplementary Figure S3C). The Venn diagram results are shown in Fig. 3D. Analysis of common differential genes among the combinations revealed that there were 138 and 84 common genes in 168-R$^-$M$^-$, compared with 168 and 168-R$^-$ respectively. But these were not significant and thus could not be subjected to KEGG enrichment. The 277 common differential genes between 168-R$^-$ and 168 were concentrated in the RNA degradation pathway (GeneRatio: 8/90) (Figure S3F).

Next, a comparative analysis of the differential genes in the three strains under the two cultivation conditions was conducted. The starting strain 168 had 179 unique differential genes, which were enriched in the β-lactam resistance pathway (GeneRatio: 6/53) according to KEGG analysis; this pathway produces β-lactam antibiotics that inhibit the formation of peptidoglycan in the bacterial cell wall. Strain 168-R$^-$ had 181 unique differential genes, but enrichment analysis showed these differences were not significant. Strain 168-R$^-$M$^-$ had 245 unique differential genes; interestingly, 10 of these genes were significantly

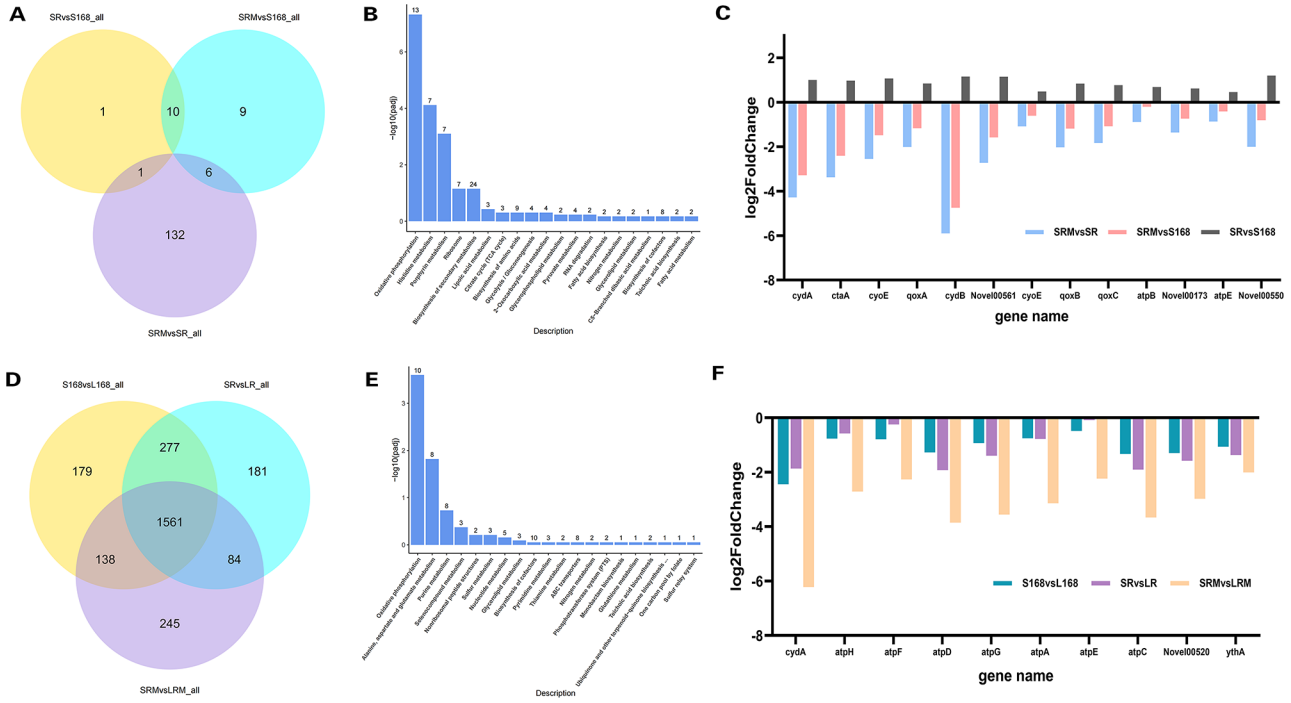


Fig. 3 Transcriptomic analysis of differential genes and KEGG enrichment of groups "SRMvsSR" and "SRMvsLRM" Differential genes of the three strains in the Spizizen condition and LB medium were analyzed. Prefix in the sample name stood for different incubation condition: "L" meant LB incubation; "S" meant the Spizizen transformation condition. **A** the Venn plot of different genes of the three strains in the Spizizen condition. Screening criteria for differential genes under the Spizizen transformation was "|log2(FoldChange)| > 0,padj < 0.05". **B** the KEGG enrichment of the independent differential genes in "SRMvsSR", padj < 0.05. **C** the specific expression fold changes of enriched 13 genes in the oxidative phosphorylation pathway. **D** the Venn plot of different genes of each strain under the Spizizen transformation and LB cultivation. Screening criteria was "|log2(FoldChange)| > 2, padj < 0.05". **E** the KEGG enrichment of the independent differential genes in "SRMvsLRM", padj < 0.05. **F** the specific expression fold changes of enriched 10 genes in the oxidative phosphorylation pathway

concentrated in the oxidative phosphorylation pathway (GeneRatio: 10/63) (Fig. 3E). The fold changes of these genes under different cultivation conditions are shown in Fig. 3F, and their functions are listed in Table 1, including some noteworthy new genes.

Most of the differential genes in the three strains under two cultivation conditions (except for *cydA* and *ythA*) were the encoding genes for subunits of ATP synthase, indicating that ATP synthase was inhibited in cells treated with the Spizizen transformation method. The inhibition was more pronounced in the strain with the deleted BsuMI methylation subunit. This result answered the question raised in the former section: the oxidative phosphorylation pathway is inhibited during the competence formation process, and the deletion of the BsuMI methylation subunit exacerbates this inhibition. Most of the differential genes of strains 168-R^-M^- and 168-R^- under the Spizizen cultivation condition encoded subunits of cytochrome ubiquinol oxidase, and the deletion of the BsuMI methylation group significantly reduced the expression levels of these genes. Therefore, we speculate that there is a close relationship between the BsuMI methylation subunit and cytochrome bd ubiquinol oxidase, which requires further in-depth studies to explore the regulatory mechanisms involved.

Common differential genes of the three strains under two cultivation conditions

To further elucidate the mechanism of competence formation, we analyzed the common differential genes of the three strains under two cultivation conditions. A total of 1561 common differential genes were identified as shown in Figure S3G, revealing that the differential genes were primarily concentrated in pathways related to nutrient metabolism and cell signal transduction, such as starch and sucrose metabolism (GeneRatio: 39/544) and the phosphotransferase system (PTS, GeneRatio: 23/544). The expression levels of these differential genes in each strain were shown in the heatmap of Fig. 4A and B. Compared to LB cultivation conditions, in the Spizizen transformation medium containing glucose, the negative regulatory element to the starch and sucrose metabolism pathway, *sacX* showed high expression levels in all three strains, while the expression levels of other genes were reduced. This phenomenon aligns with the bacterial preference for glucose. During this time, glycolysis levels in the cells were normal, but the oxidative phosphorylation

Table 1 Comparative genes enriched in oxidative phosphorylation pathway

Gene id	Gene name	SRM vs. SR log2FoldChange	SRM vs. LRM log2FoldChange	Gene description
BSU_RS20875	cydA	-4.274444115	-6.220847533	cytochrome ubiquinol oxidase subunit I
BSU_RS08175	ctaA	-3.371630073	/	heme A synthase CtaA
BSU_RS06675	cyoE	-2.546876811	/	heme o synthase
BSU_RS20565	qoxA	-2.007419883	/	cytochrome aa3 quinol oxidase subunit II
BSU_RS20870	cydB	-5.895550912	/	cytochrome d ubiquinol oxidase subunit II
BSU_RS08180	cyoE	-1.083354764	/	heme o synthase
BSU_RS20560	qoxB	-2.019840676	/	cytochrome aa3 quinol oxidase subunit I
BSU_RS20555	qoxC	-1.830885846	/	cytochrome aa3 quinol oxidase subunit III
BSU_RS19900	atpB	-0.883267492	/	F0F1 ATP synthase subunit A
BSU_RS19895	atpE	-0.86423085	/	F0F1 ATP synthase subunit C
BSU_RS19885	atpH	/	-2.711189637	F0F1 ATP synthase subunit delta
BSU_RS19890	atpF	/	-2.261211762	F0F1 ATP synthase subunit B
BSU_RS19870	atpD	/	-3.848939638	F0F1 ATP synthase subunit beta
BSU_RS19875	atpG	/	-3.554676478	ATP synthase F1 subunit gamma
BSU_RS19880	atpA	/	-3.14028338	F0F1 ATP synthase subunit alpha
BSU_RS19895	atpE	/	-2.229717615	F0F1 ATP synthase subunit C
BSU_RS19865	atpC	/	-3.66187146	F0F1 ATP synthase subunit epsilon
BSU_RS16565	ythA	/	-2.00910604	cytochrome ubiquinol oxidase subunit I
Novel00561	Novel00561	-2.723729381	/	Cytochrome bd terminal oxidase subunit I
Novel00173	Novel00173	-1.356427994	/	Glucuronate isomerase
Novel00550	Novel00550	-2.005109132	/	Cytochrome C oxidase subunit II, transmembrane domain
Novel00520	Novel00520	/	-2.974151119	ATP synthase alpha/beta family, nucleotide-binding domain

Note gene functions and pathways were analyzed based on KEGG (Kyoto Encyclopedia of Genes and Genomes) and NCBI (National Center for Biotechnology Information) database

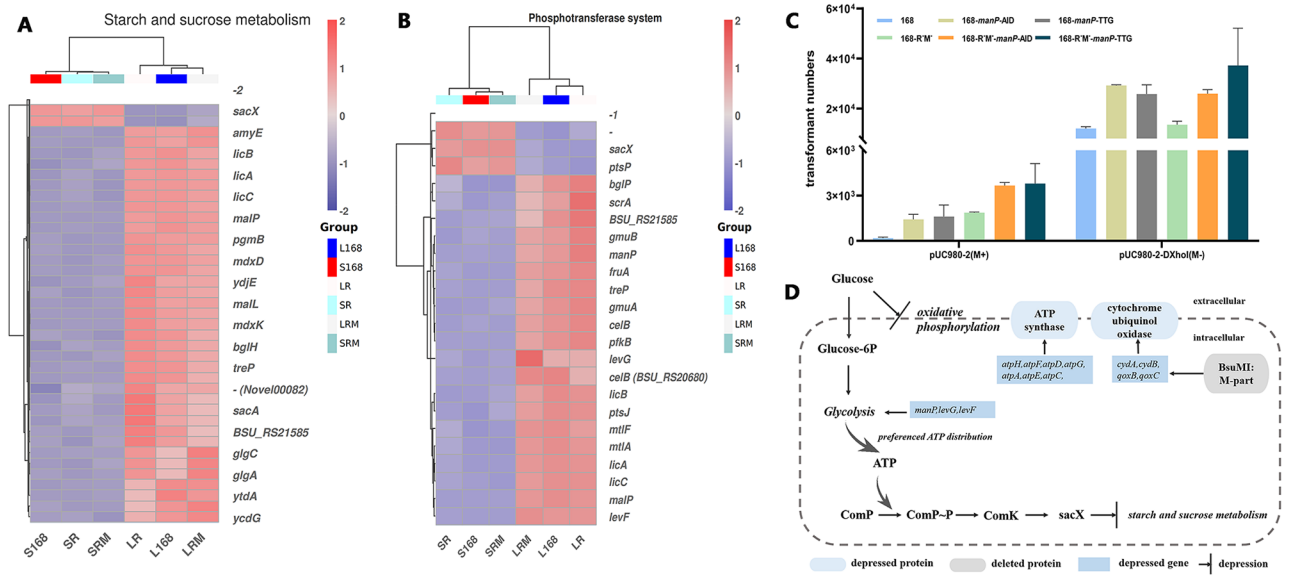


Fig. 4 Putative mechanism of competence formation and BsuMI regulation during the Spizizen transformation Combining the analysis results about the differential genes in the three strains, we proposed the metabolic flow and the regulatory role of the BsuMI methylation subunit during the competence formation process. **A** the heatmap of different genes enriched in the starch and sucrose metabolism in three strains under the two cultivation conditions. **B** the heatmap of different genes enriched in the phosphotransferase system in three strains under the two cultivation conditions. **C** plasmids transformations in constructed strains with silenced *manP* expression. All experiments were conducted with three biological replicates. $p < 0.05$. **D** mechanism of competence formation and BsuMI regulation during the Spizizen transformation

pathway was weakened, indicating that the cells chose the glycolysis pathway to consume glucose and provide the necessary energy for cell growth. Under the Spizizen cultivation conditions, most of the differential genes concentrated in the phosphotransferase system (PTS) showed down-regulated expression, including *manP*, *malP*, and *levG*, which encode phosphate group transport proteins. The decreased expression of these genes suggested that a significant amount of phosphate groups was being directed to other metabolic pathways, reflecting active phosphate signal transduction during the competence formation process.

Mechanism of competence formation and BsuMI regulation in the spizizen transformation method

Based on these analysis results, we concluded that the mechanism of competence formation of *B. subtilis* in the Spizizen transformation and BsuMI regulation pathway could mainly be divided into two aspects as illustrated in the Fig. 4D: the preferential selection of carbon sources by the cells and the preference for specific metabolic pathway when utilizing the carbon source.

In this study, cells utilized the glycolytic pathway to metabolize glucose from the environment. A sufficient supply of glucose reduced the demand for other phosphorylated precursors in this pathway, which was reflected in the transcriptional data as a decreased expression level in encoding-genes of specific phosphotransferase proteins necessary for the synthesis of these precursors, such as genes *manP* and *levG* in the PTS system. The weakening of these ATP-substrate competitive metabolic pathways allowed more ATP substrates to be directed toward the auto-phosphorylation of the signal transduction factor ComP during competence formation, producing more ComP ~ P molecules that promoted the phosphorylation of the ComA protein, thereby increasing the expression level of the key regulatory protein ComK involved in competence forming process. The expression of ComK upregulated the expression of the negative regulator SacX of the sucrase synthesis operon antiterminator SacY [29], which inhibited the metabolic pathways of starch and sucrose in host cells, reinforcing the preference for glucose as the primary carbon source.

Previous studies have shown that in bacteria, reducing the decrease in intracellular ATP synthesis caused by repressed oxidative phosphorylation levels could significantly increase the glycolytic rate of cells, compared to strategies aimed at increasing the expression of key enzymes in the glycolytic pathway [30, 31]. Through transcriptomic analysis, we have determined that the methylation modification group of the primary protein BsuMI in the restriction-modification system was indeed involved in regulating the process of competence formation. The methylation modification group functioned independently of the restriction-modification components and was associated with the functional modification of key enzymes in the oxidative phosphorylation pathway. The absence of the BsuMI methylation modification group resulted in a decrease in the expression of subunits of cytochrome oxidase, leading to a weakening of the oxidative phosphorylation pathway, which promoted the glycolytic rate of cells and subsequently affected the distribution of ATP molecules in the metabolic flux.

In all three strains, most phosphate transport proteins showed lower expression in Spizizen medium compared to conventional LB cultivation as shown in Fig. 4B. We hypothesized that reducing the expression of these proteins in the host strain might enhance transformation efficiency. To test this hypothesis, we silenced the *manP* gene in both the initial strain 168 and the restriction-modification system-deficient strain 168-R^-M^- using two strategies (premature translation termination and substitution with a low-efficiency start codon) as shown in Fig. S1C. Growth curves of recombinant strains with depressed *manP* expressions were shown in Fig. S4. By comparing transformation efficiencies, we concluded that silencing the *manP* gene increased the ability of the host strain to uptake plasmids, with the improved transformation efficiency of $1.6\times10^3\pm763$ in pUC980-2(M+) and $2.9\times10^4\pm283$ in pUC980-2-DXhoI(M-) approaching that of the restriction-modification system deficiency as illustrated in Fig. 4C. The inhibition of the *manP* gene enhanced plasmid transformation efficiency in restriction-modification system-deficient strains, independent of whether the plasmid possesses restriction-modification. The previously observed phenomenon where BsuMI methylation modification group inhibited plasmid transformation through specific recognition sites (Fig. 2D) has disappeared. This indicated that the enhancement effect of the *manP* silencing strategy is independent of the restriction-modification system. However, the combined effect of multiple strategies was limited and did not further improve plasmid transformation efficiency as expected.

Construction of a transformation platform for wild Bacillus strains based on the 168-R^{-M}

Plasmids naturally present in wild-type strains exhibit superior performances compared to commonly used vectors in the *Bacillus* system [32–34]. However, most of these plasmids are not shuttle vectors and therefore cannot be constructed in *E. coli* and subsequently transferred to the host strain according to the conventional molecular biology method. This characteristic limits the application of these wild-type plasmids. The restriction-modification system-deficient strain 168-R^-M^- constructed in this study demonstrated good operability and

high transformation efficiency, making it an ideal strain for constructing non-shuttle recombinant plasmids. Thus, the strain 168-R$^-$M$^-$ was used as a model for simulating the environment of DNA methylation modification in wild *Bacillus* strains.

Relevant methyltransferase encoding genes (Fig. 5A and B) in two wild-type *Bacillus* strains, *B. amyloliquefaciens* 205 and *B. licheniformis* 702, from our laboratory stock were used as representative genes for methylation modification originated from wild-type *Bacillus.* The *Bacillus* methylation mimicking pattern containing two recombinant strains 168-R$^-$M$^-$-P43-205Ms and 168-R$^-$M$^-$-P43-702Ms were constructed (Fig. 5C) and the strains have shown significant superiority over the control strain 168-R$^-$M$^-$ and the parent strain 168 in the late exponential growth stage until the stationary period (Fig. 5D).

Eight wild *Bacillus* strains with stored application potentials, classified into three *Bacillus* species of *B. amyloliquefaciens* (BA), *B. licheniformis* (BL), and *Bacillus tequilensis* (BT), were selected to be transformed with plasmid treated by this mimicking pattern. Compared with the controlling group DH5α and EC135, the plasmids simulated by the methylation modification environment were not only able to transform a variety of strains, but the transformation efficiencies were also greatly improved. The plasmid treated with 702Ms simulation obtained the best species universality and was successfully transformed into almost all alternative strains with the highest improvements up to 31.5-fold and 63-fold of the groups pUC980-2 (DH5α) and pUC980-2 (EC135) as shown in Fig. 5E. In the electronic transformation of BA-205, pUC980-2(205Ms) was not only successfully transformed but also accompanied by the highest efficiency, which was 19.5 and 65 times of the control group

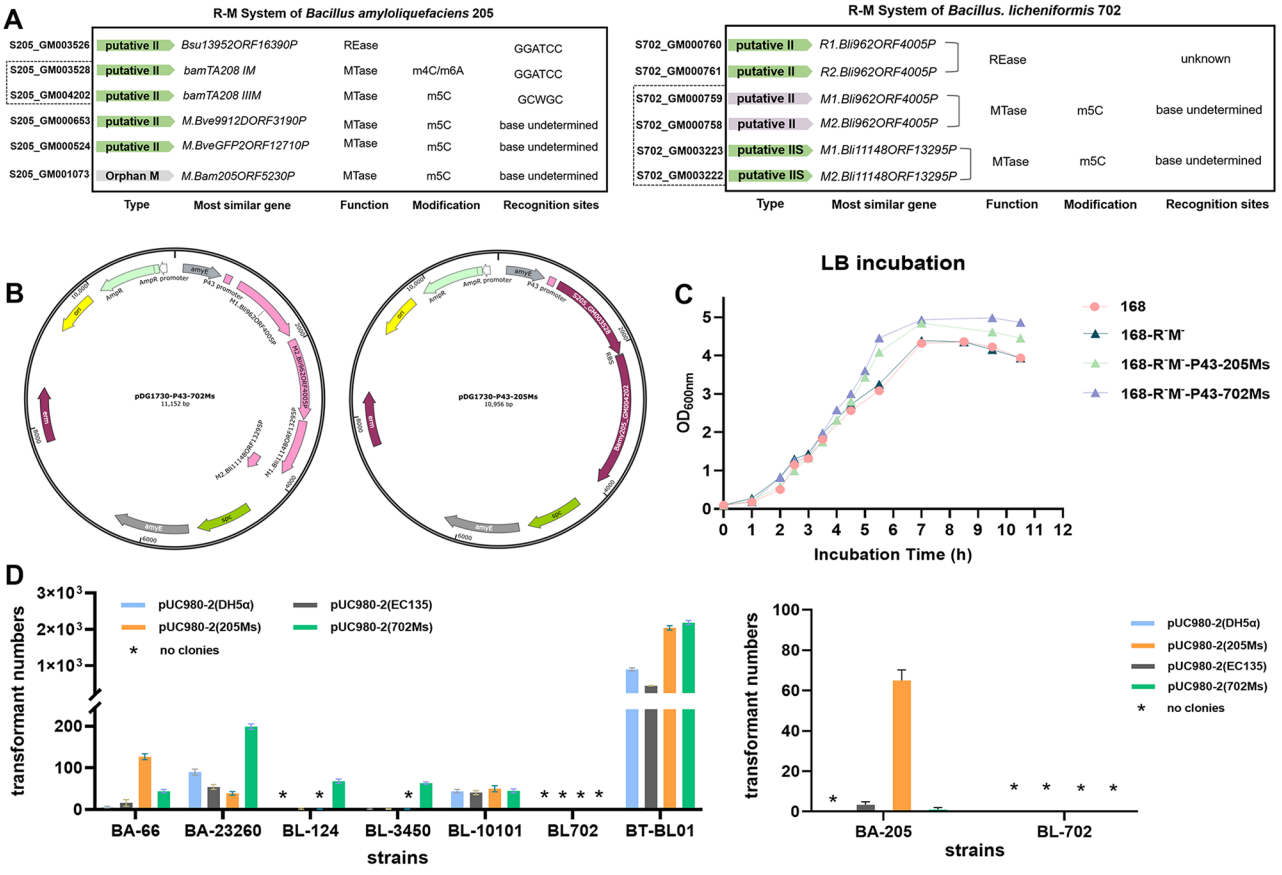


Fig. 5 Construction of a transformation platform for wild *Bacillus* based on DNA methyl-modification mimicking models The recombinant strain *B. subtilis* 168-R$^-$M$^-$ with inefficient R-M system was used to simulate DNA methyl-modifications in wild *Bacillus* strains and an efficient genetic transformation platform was established based on these simulation models. **A**: analysis of R-M systems of *Bacillus. amyloliquefaciens* 205. **B**: analysis of R-M systems of *Bacillus. licheniformis* 702. **C**: plasmids maps used for constructions of DNA methyl-modification mimicking models. **D**: growth curves of DNA methyl-modification mimicking model strains. **E**: genetic transformations in wild *Bacillus* strains. pUC980-2 obtained from different host strains representing different methylation modifications were used to transfer wild-type *Bacillus* strains. pUC980-2(DH5α) (possessed a conventional DNA methylation modification in *E. coli* DH5α), pUC980-2(EC135) (commonly used strain *E. coli* EC135 to methyl-modified plasmids for wild-type *Bacillus* transformations), pUC980-2(205Ms) and pUC980-2(702Ms) obtained from recombinant strains 168-R$^-$M$^-$-P43-205Ms and 168-R$^-$M$^-$-P43-702Ms (methylation modification environment mimicking patterns subjected to wild *Bacillus*). All experiments were conducted with three biological replicates. $p < 0.05$ in all groups

pUC980-2(EC135). In addition, all transformations with different methods failed in the strain BL-702 as stated as "no colonies" in Fig. 5E and it might be related to the strain characteristics of very tight block for foreign DNAs [35, 36].

Discussion

Bacteria preferentially select glucose from the environment as a carbon and energy source [37]. The medium used in the Spizizen transformation method contains an abundance of glucose [38]. In this study, *B. subtilis* competence cells suppressed the metabolic pathways of starch and sucrose to prioritize the utilization of glucose as concluded in Fig. 4. When the ComK protein accumulated to a certain level within the cell, it promoted the expression of SacX [39], which exerted a feedback inhibitory regulation on the starch and sucrose metabolic pathways. Comparative transcriptomic analyses in this study showed that the encoding genes for several phosphotransferase proteins were expressed at low levels during competence formation as stated in Fig. 4B. Among these, the *manP* gene encodes D-mannose phosphotransferase, which transfers a phosphate group to mannose to generate mannose-6-phosphate [40], while the products of *levG*/*levF* are responsible for converting fructose to fructose-1-phosphate [41]. These products are precursors in the glycolytic pathway, and the attenuation of their synthesis pathways was considered as a result of the abundant supply of glucose precursors in the cell and reflected the diversion of ATP toward the competence forming process.

In *B. subtilis* strains with defects in the restriction-modification system, the inhibited oxidative phosphorylation pathway reflected the cells' preference for glycolytic metabolism as concluded in Fig. 3C and F. We speculated that the reduction in oxidative phosphorylation levels might be involved in the regulation of intracellular ATP concentration. ATP is an exceptionally important co-factor in cellular activities, and its concentration regulation and distribution within the metabolic flux is a complex and intricate process [42] that merits further investigation.

In addition, the tendency of *B. subtilis* to utilize the glycolysis pathway over the oxidative phosphorylation pathway to exploit glucose is somewhat similar to the Warburg effect observed in cancer cells [43]. Competence is inherently a special state of the cell, and the absence of the restriction-modification system, which is considered as a defense line of bacterial immune system [44, 45], makes the cells more abnormal. In strains with defects in the restriction-modification system, the lack of the BsuMI methylation modification group affected the function of cytochrome oxidase, leading to a weakening of the oxidative phosphorylation pathway [46]. The reliance on the glycolytic pathway and the preferential distribution of ATP in the metabolic flux was thought to be an efficient strategy employed by cells to meet the requirements for growth and differentiation under special conditions [47, 48].

The activation mechanism of competence formation in *B. subtilis* cells discussed in this study was dependent on the distribution of ATP within the metabolic flux. A significant amount of ATP, which would otherwise be used in other metabolic pathways, was redirected to the signal transduction pathways involved in competence forming process due to the cell's metabolic preference in this special state, making the host bacteria more receptive to exogenous DNA. This also explained why reducing ATP utilization in other metabolic pathways (such as in *manP* gene-silenced strains) could also enhance the cell's ability to take up exogenous DNA as shown in Fig. 4C. However, the effectiveness of this "ATP snatching" strategy was determined to be limited as the combination of multiple strategies did not lead to a multiplicative increase in transformation efficiency.

It is important to note that the mechanism hypothesis derived in this study was limited by the constraints of transcriptomic analysis, which only represented regulation at the RNA level. It did not capture differences in enzyme activity and metabolic flux at the translational level, which is required to obtain more precise conclusions in further analyses. Moreover, the plasmid transformation results suggested that the BsuMI methylation subunit did restrict plasmid transformation into *B. subtilis*. Our current work results concluded that the restrictive effect of the BsuMI methylation subunit was independent of the plasmid's methylation status and related to the target site sequence carried by the plasmid. Meanwhile, an unexpected decrease in transformation efficiencies was observed along with the deletion of recognition site from plasmid pUC980-2(M-) pUC980-2-DXhoI(M-) in strains 168-R^- and 168-R^-M^-(Fig. 2D), while these opposite results were not observed in transformations of pHT43-DXhoI(M-) (Fig. S2). Considering the different replication types and copy numbers of the pUC980-2 and pHT43 plasmids, it was inferred that this difference was related to plasmid replication. Further investigations deep into DNA adsorption system are needed to clarify the mechanism that involves the plasmid entry process.

The important role of the R-M system in *Bacillus* strains was also demonstrated by the recovery and even better growth states of 168-R^-M^-(Fig. 5D), which was consistent with the previous studies [49]. Plasmids transformation improvements in wild *Bacillus* based on the methylation mimicking patterns were obtained not only in strains of the same species from which the methyl simulations originated, but also in strains of different species,

indicating non-preference and universality of this platform (Fig. 5D). This platform was believed to be a reliable technical support for genetic manipulations and chassis cell developments with *Bacillus* strains.

Methods

Strains, plasmids and materials

Strains and plasmids used in this study are listed in Table S1. *E. coli* DH5α was used for plasmids constructions and amplifications. *E. coli* 110 was hired to prepare plasmids with non-methylation modifications. *E. coli* EC135 containing methyltransferase expressing plasmid pBM was built previously according to the publication of Zhang G et al. [28]. *B. subtilis* 168 was manipulated to construct strains with inefficient restriction-modification systems and their derivative strains for mechanism validations. The transcriptomic sequencing of strains *B. subtilis* 168, *B. subtilis* 168-R^- and *B. subtilis* 168-R^-M^- were carried out by Novogene Co., Ltd (Beijing, China). Genome sequences of wild strains *B. amyloliquefaciens* 205 and *B. licheniformis* 702 were screened for methyltransferase genes and used in DNA-methylation mimicking patterns. Genomic sequencing of *B. amyloliquefaciens* 205 and *B. licheniformis* 702 were performed by Novogene Co., Ltd. Other wild type strains *B. amyloliquefaciens* 66, *B. amyloliquefaciens* 23,260, *B. licheniformis* 3450, *B. licheniformis* 124, *B. licheniformis* 10,101 and *Bacillus tequilensis* BL01 were all stored in our lab and stated in Table S1. The restriction endonucleases and high-fidelity DNA polymerase PrimeSTAR Max for gene amplifications were commercially supplied by TaKaRa Bio Co., Ltd (Dalian, China). The Easytaq DNA polymerase Hieff PCR Master Mix, plasmids extraction kit and bacterial whole genome extraction kit were purchased from Yeasen Biotechnology Co.,Ltd (Shanghai, China). All other enzymes, chemicals and reagents were purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). All the DNA sequencing for plasmids and strains confirmations were performed in GENEWIZ Biotechnology Co.,Ltd (Suzhou, China).

Cultivation methods

Cells were routinely grown in Luria-Bertani (LB) medium. Growth curves of recombinant strains were determined by measuring the optical densities at 600 nm (OD_{600nm}) in LB medium. Different antibiotics (25 μg/mL kanamycin, 5 μg/mL chloromycetin and 200 μg/mL spectinomycin). 0.8 mM IPTG was added into the medium for inducement of CRISPR editing.

Cells in Spizizen transformations were incubated in GM1 medium (5 mL total volume contains 100 μL 40% glucose, 100 μL 2% acid hydrolysis tyrosine, 100 μL 5% yeast extraction, 5 μL 20% $MgSO_4{\cdot}7H_2O$, 500 μL 10x spizizen salt medium, 50 μL 0.5% L-tryptophan and complemented double distilled water) and GM2 medium (5 mL total volume contains 100 μL 40% glucose, 50 μL 2% acid hydrolysis tyrosine, 35 μL 20% $MgSO_4{\cdot}7H_2O$, 500 μL 10x spizizen salt medium, 5 μL 0.5% L-tryptophan and complemented ddH_2O). 100 mL volume of 10x spizizen salt medium (pH 7.2) contains 2 g $(NH_4)_2SO4$, 18.3 g K_2HPO_4, 6 g KH_2PO_4, 1.2 g sodium citrate and complemented water.

Wild strains during electric transformations were incubated in the growth medium LBS (9 g sorbitol per 100 mL LB medium) and washed with the medium SHMP containing 0.25 M saccharose, 1 mM $MgCl_2$ and 10% glycerol. Cells after electric treatments were grown in the recovery medium LBSPG composing of LB with 0.25 M saccharose and 10% glycerol.

All strains were cultured in tubes and shake flasks (SHUNIU, Sichuan SHUBO Co., LTD, China), incubated at 37℃ and 200 rpm (unless mentioned specifically) in a rotary shaker (ZQWY-200G, Shanghai Zhichu Instruments Co., Ltd., China).

Plasmid constructions

Primers used for plasmids constructions and PCR verification were listed in Table S2. Plasmid pHT43-cas9 and pUC980-7 constructed in our previous studies [50, 51] and their improved versions pHT43-cas9-GT-DBsaI, pHT43-AID-spec, pUC980-7-GT-DBsaI and pcrF-N20 were used to construct CRISPR plasmids in this work. RA-N20-S13, M2-N20-S15, manP-N20-ATG and manP-N20-S96 were the target-N20s on genes *bsuRA*, *bsuMB* and *manP* separately. Among them, manP-N20-ATG was used for the transversion from the start codon "ATG" to "TTG" and manP-N20-S96 was targeted for the transition from "CAA" to "TAA" to terminate gene expression prematurely.

Primer-pair oligos P1/P2 and P9/P10 were annealized and inserted into corresponding sites on vectors through the BsaI Golden Gate protocol, resulting plasmids pHT43-cas9-RAN20S13, pUC980-7-M2N20S15. The flanking arms of gene cluster *bsuRABC* and *bsuMAB* were amplified with primer pairs P3/P4, P5/P6 and P11/P12, P13/P14. The resulted fragments with corresponding sizes of 1258 bp, 1353 bp and 1211 bp, 1131 bp were ligated with the SfiI-digested fragment of pHT43-cas9-RAN20S13 and pUC980-7-M2N20S15 by the Gibson Assembly method, producing plasmids pHT43-cas9-RAN20S13-DRtemp and pUC980-7-M2N20S15-DMtemp for *bsuRABC* and *bsuMAB* deletions (Fig. S1A). The primer pair P17/P18 was used to amplify vector pcrF-N20 and the purified PCR product (5713 bp) was further transferred into *E. coli* DH5α to obtain plasmid pcrF-manPN20. Sequences flanking the starting codon of gene *manP* were amplified with primers P19/P20 and P21/P22. Resulted fragments of 536 bp and 533 bp were

purified and inserted into the vector pcrF-manPN20 through the BsaI Golden Gate protocol, producing plasmid pcrF-manPN20-temp for "ATG to TTG" transversion. The primer pair P23/P24 was annealed and inserted into vector pHT43-AID-spec through the BsaI Golden Gate protocol. The resulted plasmid pHT43-AID-spec-manPN20S96 was further used for "CAA to TAA" transition.

The primer pair P27/P28 was used to amplify the P43 promoter and its coherent RBS sequence from plasmid pUC980-2, an expressing vector constructed in our previous study based on pWB980 [51]. Resulted fragment (220 bp) was ligated through overlap PCR with the methyl-transferase genes S205_GM003528 (1318 bp) and S205_GM004202(1860 bp), amplified from the *B. amyloliquefaciens* 205 genome by primers P29/P30 and P31/P32. The ligated fragment and the PCR product of pDG1730 (7600 bp) with primers P33/P34 were further assembled to integrate plasmid pDG1730-P43-205Ms. Combining the BsaI Golden Gate protocol and overlap PCR, the P43-RBS fragment (230 bp) amplified with primers P27/P35 from pUC980 was ligated with gene clusters originated from the *B. licheniformis* 702 genome, i.e. S702_GM000758-0759 (primer pair P36/P37, 2246 bp) and S702_GM003222-3223 (primer pair P38/P39, 1076 bp). The ligated fragment was further assembled with the fragment of pDG1730 amplified with primers P33/P34, resulting plasmid pDG1730-P43-702Ms.

Plasmids pUC980-2 and pHT43 were hired during determinations of transformation efficiencies to recombinant strains in this study. In order to eliminate the possible regulation sites recognized by the restriction-modification system of *B. subtilis* 168, the specific sequences "CTCGAG" on plasmids pUC980-2 and pHT43 were deleted with primer pairs P42/P43(4225 bp) and P46/P47(8072 bp) respectively. The deletions were further verified and confirmed with primers P44/P45 and P48/P49 for plasmids pUC980-2-DXhoI and pHT43-DXhoI.

Plasmid transformations

In this work, the Spizizen transformation method was used during transformant determinations and comparisons of recombinant strains with certain improvements [18]. The frozen bacterial samples were refreshed on LB solid medium after overnight incubation at 37℃. Single colonies were selected to be cultured in 5 mL GM1 medium at 37℃, 200 rpm. After culturing for 14 h, 500 μL of each GM1 bacterial solution was injected into 4.5 mL fresh GM1 to grow at 37 ℃, 200 rpm for 4.5 h, then 500 μL GM1 mixture was transferred into 4.5 mL GM2 medium for further incubation of 1.5 h at 37 ℃, 200 rpm. The competence cells were prepared and used currently. Every competence-cell dose of 1 mL was transferred with 1 μg plasmid DNA and the mixtures were cultured at 37℃ (the temperature was dependent on the plasmid-type), 200 rpm for at least 3 h till the OD_{600nm} reached to 1.0, which was an important standardization for determinations of transformation efficiencies. Mixtures of plasmids and cells were spread on selective plates with appropriate dilutions. Transformant numbers were countered and used to evaluate the transformation efficiencies as the CFU of all experimental strains with R-M constructions in 1 mL GM2-incubation liquid (OD_{600nm}: 1.0) were examined to be located in the range of 5.9×10^8-8.7×10^8. Due to this accordance, counting numbers of transformants were used to present the transformation efficiencies in this study.

Electronic transformation method was hired for plasmids transformations of wild-type *Bacillus* strain 205 [50]. Bacterial colonies were transferred into LB medium for overnight growing at 37℃, 200 rpm. 2% inoculates were injected into LBS growth medium and cultured at 37℃, 200 rpm in baffled conical flasks. When the OD_{600nm} reached to 0.85–0.95, the culturing liquid of 40 mL was collected into 50 mL centrifuge tubes for 20 min ice-bathes and cells were harvested with centrifugation at 12,000 g, 4℃ for 10 min. After decanting the supernatant gently, the cells were re-suspended with 25 mL SHMP wash-buffer and centrifuged again to re-collect cells. This washing treatment was repeated for three times and 2 mL wash-buffer was used to suspend cells. Mixtures were dispensed into 1.5 mL EP tubes with 100 μL per tube and store at -80℃. Every dose of competence cells was transferred with 1 μg plasmid DNA and incubated with ice-bath for 20 min. Mixtures of cells and plasmids were added into pre-chilled 2 mm electroporation-cuvettes to be shocked at 1500v-2000v pulse generated by a BTX ECM399 electroporator (Harvard Apparatus). The mixtures were then suspended with 900 μL cold recovery-medium LBSPG immediately. These mixtures were further cultured at 37℃, 200 rpm for at least 3 h and spread on selective plates after treatments at 46℃ for 8 min. Colony numbers were countered and compared.

Except for special cases, all experiments were conducted with three biological replicates. The significance of differences in experimental results, unless otherwise specified, was determined at $p<0.05$ calculated by GraphPad Prism.

Genomic editing and gene integration

CRISPR/Cas9 editing and plasmid elimination experiments in this study were carried out. Deletions on the genome of constructed strains *B. subtilis* 168-R^- and *B. subtilis* 168-R^-M^- were confirmed by PCR amplifications with primer pairs P7/P8 (non-editing: 6421 bp, deleted: 3016 bp, 3405 bp was deleted) and P15/P16 (non-editing: 5466 bp, deleted: 2991 bp, 2475 bp was

deleted) respectively as shown in Fig. S1B. Base transversions on the genomes of *B. subtilis* 168-*manP*-TTG and *B. subtilis*168-R⁻M⁻-*manP*-TTG were verified by DNA sequencing results with the primer pair P19/P26(Fig. S1C). The nucleotide-base transitions mediated by CRISPR/AID were conducted through similar protocols to CRISPR/Cas9, except that correct editing results were confirmed to show double peaks at target sites illustrated by DNA sequencing with the primer pair P25/P26 and further selections were required to obtain homozygote strains *B. subtilis* 168-*manP*-AID and *B. subtilis*168-R⁻M⁻-*manP*-AID(Fig. S1C). All the target sequences N20 of CRISPR/Cas9 systems used in this work were designed at websites http://targetfinder.flycrispr.neuro.brown.edu/ and http://crispr-era.stanford.edu/index.jsp/. Target N20 sites for CRISPR/AID were designed at the website http://gbig.ibiodesign.net/index.html.

Gene-clusters coding methyltransferases were fused with the vector pDG1730, which is unable of self-replicating in *Bacillus* cells. Through the homologous recombination of single cross-over mode, aimed genes were inserted into the commonly used site *amyE* on the genome of *B. subtilis* R⁻M⁻. The whole genomic DNAs of resulted strains *B. subtilis* 168-R⁻M⁻-P43-205Ms and *B. subtilis* 168-R⁻M⁻-P43-702Ms were confirmed by PCR amplifications with the primer pair P40/P41 (205Ms integration: 3786 bp, 702Ms integration: 3982 bp) as shown in Fig. S1D.

Transcriptomic analysis

In the Spizizen transformation method, cells were first inoculated into GM1 medium and grown for 4.5 h, then transferred to GM2 medium for 1.5 h incubation before harvest. Cells in LB incubation were harvested after 4.5 h. The transcriptomic data of the competence formation process for each strain was collected by the Novogene Co., Ltd. Clean reads were aligned to the *B. subtilis* 168 genome (Genbank accession number NC_000964.3) using Bowtie2. Prefix in the sample name stood for different incubation conditions: "L" meant LB incubation; "S" meant the Spizizen transformation condition. Samples L168_2 and LR_1, which had R^2 values less than 0.8 with the other two biological replicates, were excluded to guarantee the correlation was above 0.8. Thus, groups L168 and LR contained only two replicates and others had three replicates. Raw data has been submitted to the SRA database of NCBI with a BioProject accession number PRJNA1133204.

Deferentially expressed genes between two samples were significantly analyzed with software DESeq2 with biology replicates. Screening criteria for differential genes in strains during the Spizizen transformation or LB medium was "|log2(FoldChange)| > 0, padj < 0.05". For those between the Spizizen Transformation and LB Cultivation, the standard was "|log2(FoldChange)| > 2, padj < 0.05". Further comparative transcriptome analyses were conducted by the online software provided by the supporting platform Novomagic (https://magic.novogene.com) of Novogene Co., Ltd. The screening criteria for heatmapping and KEGG enrichment was "padj < 0.05". The metabolic pathways were referenced to the KEGG database (https://www.genome.jp/kegg/pathway.html).

Methylation analysis

The other two methyltransferases M.BsuMIIP and BsuMORF6760P in *B. subtilis* 168 are separately encoded by the genes *mtbP* and *yeeA* separately. According to previous researches, these two genes and their encoded proteins are not involved in DNA restriction mechanism during the transformation process [52–54]. Therefore, this study was mainly focused on BsuMI to investigate the regulatory pathway of R-M system on DNA transformation process in *Bacillus* cells.

The genomes of *B. amyloliquefaciens* 205 (NCBI number: CP054415) and *B. licheniformis* 702 were screened and compared based on the restriction enzyme database REBASE http://rebase.neb.com/rebase/rebase.html with a localization. Methyltransferases coding gene clusters stated as 205Ms and 702Ms were applied in methyl-modification mimicking patterns. 205Ms consist of genes S205_GM003528 and S205_GM004202 with 100% similarities of genes *bamTA208 IM* and *bamTA208 IIIM* (NCBI number: NC_017188.1) respectively. 702Ms contain genes S702_GM000758, S702_GM000759, S702_GM003222, S702_GM003223, the same with *M1.Bli962ORF4005P*(NCBI number: NCTC962_04005), *M2.Bli962ORF4005P*(NCBI number: NCTC962_04006), *M1.Bli11148ORF13295P*(NCBI number: D9Y32_13295) and *M2.Bli11148ORF13295P*(NCBI number: D9Y32_13300) correspondingly.

Abbreviations

R-M system	Restriction-modification system
REase	Restriction endonuclease
MTase	Methyltransferase
AdoMet	S-adenosylmethionine
IPTG	Isopropyl-β-D-thiogalactoside
PTS	Phosphotransferase system

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02493-z.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Acknowledgements
We are grateful for the help of Miss Gu Rongrong during the localization of REBASE database for methyltransferase screening. And we really appreciate the inspired discussions with Dr. Shu Wenju.

Author contributions
The research was designed and planned by Zhao Xingya, Zheng Hongchen, Bai Wenqin and Song Hui. Zhao Xingya led the performance of whole project, conducted major experiments, data analyses, wrote and improved the manuscript. Fu Xiaoping conducted the construction of *E. coli* EC135 and preliminary verifications of methylation genes. Zhen Jie and Yang Jun conducted DNA extractions. Zheng Hongchen and Bai Wenqin participated in editing the paper. Song Hui, Zhao Xingya, Bai Wenqin and Zheng Hongchen provided funding-supports. All authors read and approved the final manuscript.

Funding
This research was funded by the National Key Research and Development Program of China (2021YFC2100201) (SH) and the Tianjin Synthetic Biotechnology Innovation Capacity Improvement Projects: grant number TSBICIP-CXRC-037(ZXY), TSBICIP-PTJJ-007-13(BWQ) and TSBICIP-KJGG-009-0202(ZHC).

Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Industrial Enzymes National Engineering Research Center, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin Airport Economic Area, 32 West 7th Avenue, Tianjin 300308, China
[2]Tianjin Key Laboratory for Industrial Biological Systems and Bioprocessing Engineering, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin Airport Economic Area, 32 West 7th Avenue, Tianjin 300308, China
[3]National Center of Technology Innovation for Synthetic Biology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin Airport Economic Area, 32 West 7th Avenue, Tianjin 300308, China
[4]Key Laboratory of Engineering Biology for Low-Carbon Manufacturing, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin Airport Economic Area, 32 West 7th Avenue, Tianjin 300308, China

Received: 2 March 2024 / Accepted: 29 July 2024
Published online: 09 August 2024

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