Phytopathology Research

RESEARCH

Open Access

Efficient transformation and genome editing in a nondomesticated, biocontrol strain, *Bacillus subtilis* GLB191

Yu Zhao[1], Zhenshuo Wang[1], Qian Wang[1], Bing Wang[1], Xiaoning Bian[1], Qingchao Zeng[1], Daowan Lai[1], Qi Wang[1] and Yan Li[1*]

Abstract

Bacillus subtilis has been widely used as a biological control agent in agricultural production. Environmental strains of *B. subtilis* are an important source of biological control agents. However, due to its low genetic transformation efficiency, the genetic manipulation of the environmental and nondomesticated strains is challenging. In this study, the impact of competent cell preparation, pulse electroporation, and recovery culture on the electroporation efficiency of *B. subtilis* GLB191 was assessed utilizing response surface methodology. Results indicated that the concentration of glycine, DL-threonine, and Tween 80 used in a cell wall weakening solution during competent cell preparation, and the voltage applied during pulse electroporation were the primary factors affecting electroporation efficiency. Optimization of these factors led to nearly a three-fold increase (reaching 74.00 ± 5.10 CFU/μg DNA) in electroporation efficiency. The elimination of *dam* and *dcm* modifications to mitigate the influence of host restriction-modification systems was integrated to further increase the electroporation efficacy. An electroporation efficiency for replicative plasmids of $1.96 \pm 0.05 \times 10^6$ CFU/μg DNA was achieved using the optimized strategy. Utilizing this improved methodology, the temperature-sensitive plasmid pJOE8899 was efficiently transformed into *B. subtilis* GLB191, resulting in a markerless knockout of *pdeH*. The optimized transformation strategy significantly enhances the efficiency of markerless genome editing of nondomesticated *B. subtilis*, offering the potential for future interpretation of their modes of action, which is critical for the development of the nondomesticated *B. subtilis* strains.

Keywords *Bacillus subtilis*, Electroporation, Response surface methodology, Genome editing

Background

The gram-positive bacterium *Bacillus subtilis* has been the subject of considerable research due to its ability to inhibit pathogenic microorganisms and enhance plant growth (Schallmey et al. 2004; Berg 2009). *B. subtilis* GLB191 (hence referred to as GLB191) is a nondomesticated endophyte isolated from wild grape leaves and exhibits significant biocontrol efficacy against grapevine downy mildew caused by *Plasmopara viticola* (Zhang et al. 2017). The ability to conduct efficient genetic manipulation is essential to fully exploit its mechanisms and develop GLB191 as a biological control agent. In this regard, markerless genetic engineering techniques are often utilized due to their capacity to achieve multi-locus iterative editing on the same chromosome (Dong and Zhang 2014). Therefore, an efficient genetic transformation system is essential for markerless genome manipulation in *B. subtilis*.

The efficiency of natural transformation is closely linked to specific genes, such as the competence

*Correspondence:
Yan Li
liyancau@cau.edu.cn
[1] Department of Plant Pathology, MOA Key Lab of Pest Monitoring and Green Management, College of Plant Protection, China Agricultural University, Beijing 100193, China

regulatory genes *comK* and *comS*, as well as quorum sensing related genes (Tran et al. 2000; She et al. 2020). Different expression levels of these genes result in notable variations in transformation efficiency in different *B. subtilis* strains (Tran et al. 2000; She et al. 2020). Overexpression of *comK* and *comS* increased the transformation efficacy (Deng et al. 2021). In addition, host-restricted modification systems have also been reported to affect exogenous DNA uptake (van der Rest et al. 1999; Mahipant et al. 2019; Jeong et al. 2022). The restriction-modification (R-M) system, comprising methyltransferases (MTases) and restriction enzymes (REases), is widely distributed among bacteria (Roberts et al. 2022). The R-M systems are categorized into four types (type I–IV): the type I–III recognize and cleave DNA that lacks specific methylation, while the type IV targets DNA that has undergone such modifications (Roberts et al. 2003). Similar to the CRISPR-Cas system, the R-M system is involved in bacterial immune defense. However, this defense mechanism inevitably hinders the genetic transformation of many bacterial species, making it more challenging.

Genetic transformation represents a method of introducing exogenous DNA into bacterial cells. The natural transformation method has been extensively studied and applied to domesticated laboratory strains, such as *B. subtilis* 168, which efficiently uptake exogenous DNA through natural transformation (Klein et al. 1992; Jarmer et al. 2002). However, a significant challenge arises when attempting to transform nondomesticated strains of *B. subtilis*, which have typically been documented and utilized for biocontrol. They show a much lower transformation efficiency than the laboratory strain 168, likely due to altered genetic regulation (She et al. 2020). Various transformation methods have been explored to achieve efficient genetic transformation of nondomesticated strains of *B. subtilis*, including phage transduction (Yasbin and Young 1974), protoplast transformation (Chang and Cohen 1979), and electroporation (Xue et al. 1999). However, the first two of these methods are time-consuming and exhibit low efficiency. In contrast, electroporation is a time-efficient method of transformation that has been widely used in *B. subtilis*. However, no universal protocol for efficient electroporation exists for different *B. subtilis* strains. Multiple factors, such as the type of culture medium, growth stage, electric field strength during electroporation, quantity and purity of plasmid DNA, and composition of the electroporation buffer, collectively impact the electroporation efficiency (McDonald et al. 1995; Peng et al. 2009; Zhang et al. 2011; Lu et al. 2012). Therefore, it is necessary to use a systematic multi-factorial experimental design approach to optimize the electroporation transformation efficiency of different nondomesticated *B. subtilis* strains. Response Surface Methodology (RSM) is a factorial design-based experimental and regression analysis method utilized to identify and optimize the crucial factors that maximize the response variable (Box and Wilson 1992). RSM has been used to optimize culture media formulations and production conditions in various commercial applications of microorganisms and has also been proven to be a highly effective method for optimizing electroporation conditions (Bezerra et al. 2008).

In the present study, the main factors affecting the efficacy of competent cells, electroporation, and post-recovery culture during electroporation were assessed and systematically optimized using RSM. The elimination of *dam* and *dcm* modifications to effectively eliminate the influence of host restriction-modification systems, combined with the optimized electroporation protocol, resulted in an electroporation efficiency of $1.96 \pm 0.05 \times 10^6$ CFU/μg DNA for replicative plasmids. To further confirm the improved electroporation efficiency of our protocol, the temperature-sensitive plasmid pJOE8899 was efficiently transformed into GLB191, resulting in a markerless knockout of *pdeH*. The optimized transformation strategy significantly improves the efficiency of markerless genome editing of nondomesticated *B. subtilis*, offering the potential for future interpretation of their modes of action, which is critical for the development of the nondomesticated *B. subtilis* strains.

Results

Electroporation has advantage over natural transformation for *B. subtilis* GLB191

We initially conducted experiments to determine an efficient transformation system for nondomesticated *B. subtilis* strain GLB191 to provide a foundation for genetic modification of this strain. Firstly, the transformation efficiencies of natural transformation were investigated. The results showed that neither the suicide plasmid pDG1662 nor the replicating plasmid pHT01 were successfully transformed into GLB191 using the natural transformation method (Table 1). Since Deng et al. (2021) generated "super competent" cells with enhanced transformation efficiency in *Bacillus* by overexpressing competent regulatory genes *comK* and *comS*, a combination of natural transformation with the mannitol-induced overexpression of *comK* and *comS* in plasmid pWYB108 in GLB191 were conducted to increase the transformation efficiency. The transformation efficiency of pDG1662 was 10.00 ± 0.82 CFU/μg DNA, and that of pHT01 was 22.00 ± 6.68 CFU/μg DNA (Table 1). However, leakage expression of *comK* and *comS* occurred without the addition of inducing agent mannitol, and this impacted the phenotype of GLB191, such as biofilm formation

Table 1 Transformation efficiency of GLB191

Plasmids	Natural transformation		Electroporation
	−	+	
Integrative plasmid pDG1662	0.00 ± 0.00 b	10.00 ± 0.82 a	9.67 ± 0.94 a
Replicative plasmid pHT01	0.00 ± 0.00 b	22.00 ± 6.68 a	20.67 ± 1.70 a

Transformants per μg of DNA

− indicates not overexpression of *comK* and *comS*, + indicates overexpression of *comK* and *comS* in pWYB108

Different letters on the same row indicate significant differences between different treatments according to the least significant difference (LSD) test ($p < 0.05$)

on a plate (Additional file 1: Figure S1a–c). Therefore, a kick-out of the plasmid pWYB108 is necessary before the phenotypic observation. Strains containing the pWYB108 plasmid were inoculated into non-selective LB medium and incubated at 37 °C for 9 cycles of 12 h each on a rotary shaker to kick out the plasmid and then diluted and plated on both selective and non-selective LB plates to calculate the elimination rate. The result showed that only 5.40 ± 3.83% of colonies successfully kicked out the plasmid, which is too low to use (Additional file 1: Figure S1d, Additional file 2: Table S1). Therefore, natural transformation with overexpression of *comK* and *comS* is unsuitable for subsequent phenotypic observation, although it can increase transformation efficiency. Subsequently, electroporation was used, and the transformation efficiency (pDG1662, 9.67 ± 0.94 CFU/μg DNA; pHT01, 20.67 ± 1.70 CFU/μg DNA) was comparable to that of natural transformation combining overexpression of *comK* and *comS*, and significantly higher than that of natural transformation without overexpression of *comK* and *comS*, whose transformation efficiency was 0.00 ± 0.00 CFU/μg DNA for both plasmids (Table 1). Hence, our data indicate that electroporation offers a distinct advantage when conducting transformation experiments.

Identification of factors significantly affecting electroporation efficiency

Since multiple factors collectively influence electroporation efficiency, Response Surface Methodology (RSM) was used to systematically analyze and optimize electroporation (Box and Wilson 1992). A Plackett-Burman experimental design was initially employed to identify factors that significantly impact electroporation efficiency in the processes of competent cell preparation, electroporation, and recovery culture. Nine candidate factors were selected for systematic evaluation based on initial laboratory experiments, including sorbitol concentration (*A*) in the growth medium, the concentration of glycine (*B*), DL-threonine (*C*), Tween 80 (*D*), and tryptophan (*E*) in the weakening solution, voltage (*F*) during electroporation, sorbitol (*G*), and mannitol (*H*) concentration in the electroporation solution, and the concentration of mannitol (*I*) in the recovery medium (Additional file 2: Table S2). The investigated factors were varied in the electroporation experiments based on the Plackett-Burman experimental design table, and the obtained data were compiled into a response value list and subjected to statistical analysis (Additional file 2: Table S3).

Results of the analysis of variance (ANOVA) are presented in Table 2. The experimental model yielded an *F*-value of 78.08 with a *P*-value of 0.0127, indicating that the model significantly explained the impact of the

Table 2 ANOVA of the regression model based on the Plackett–Burman experimental design

Source	Sum of squares	df	Mean square	F-value	*P*-value	Significance[a]
Model	4456.97	9	495.22	78.08	0.0127	*
A	30.08	1	30.08	4.74	0.1613	
B	412.23	1	412.23	64.99	0.0150	*
C	2591.12	1	2591.12	408.53	0.0024	**
D	710.45	1	710.45	112.01	0.0088	**
E	15.56	1	15.56	2.45	0.2577	
F	374.08	1	374.08	58.98	0.0165	*
G	12.68	1	12.68	2	0.2930	
H	234.08	1	234.08	36.91	0.0260	*
I	76.68	1	76.68	12.09	0.0737	
Residual	12.69	2	6.34			
Cor total	4469.66	11				

The R^2 and adjusted R^2 values are 0.9972 and 0.9844, respectively

[a] * and ** indicate statistical significance at $p < 0.05$ and $p < 0.01$, respectively

various tested factors. The R^2 value was 0.9972, with an adjusted R^2 of 0.9844, indicating the model design is reasonable. The *P*-values for glycine in the weakening solution (*B*), mannitol in the electroporation buffer (*H*), and voltage during electroporation (*F*) were 0.0150, 0.0260, and 0.0165, respectively, indicating a significant impact. The *P*-values for DL-threonine (*C*) and tween 80 (*D*) in the weakening solution were 0.0024 and 0.0088, indicating that these factors were highly significant. In contrast, the *P*-values for sorbitol in the growth medium (*A*), tryptophan in the weakening solution (*E*), sorbitol in the electroporation buffer (*G*), and sorbitol in the recovery medium (*I*) were 0.1613, 0.2577, 0.2930, and 0.0737, respectively, indicating that these factors were not significant in the tested ranges. These results suggest that the main factors impacting the electroporation efficiency of GLB191 are glycine, DL-threonine, and Tween 80 in the weakening solution which play vital roles in cell wall weakening and changes in cell membrane integrity during competent cell preparation, as well as the voltage used during electroporation and mannitol with the protective effect in the electroporation buffer.

The magnitude of the effect of the five significant factors on electroporation efficiency, from greatest to least, are as follows: DL-threonine (*C*), Tween 80 (*D*), and glycine (*B*) in the weakening solution, voltage (*F*) during electroporation, and mannitol (*H*) in the electroporation buffer (Fig. 1). DL-threonine, Tween 80, and glycine in the weakening solution have a significant positive effect (Fig. 1, Additional file 2: Table S4). This implies that an increase in the concentrations of these factors enhances electroporation efficiency. Conversely, increases in the concentration of mannitol in the electroporation buffer and an elevation in the voltage during electroporation have a negative effect and decrease electroporation efficiency (Fig. 1, Additional file 2: Table S4).

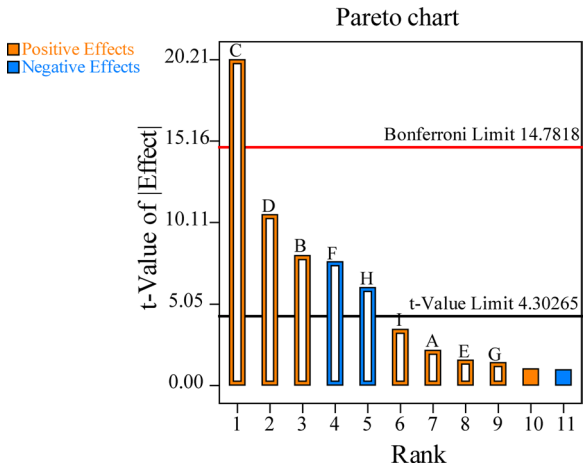

Fig. 1 Pareto chart of the factors evaluated in the Plackett–Burman experimental design. Factors including sorbitol concentration (*A*) in the growth medium; glycine (*B*), DL-threonine (*C*), Tween 80 (*D*), and tryptophan (*E*) in the weakening solution; sorbitol (*G*) and mannitol (*H*) concentration in the electroporation solution; voltage (*F*) during electroporation; and mannitol (*I*) concentration in the recovery medium are ranked based on the magnitude of their effect, with orange indicating positive effects and blue indicating negative effects on transformation efficiency. Effects above the t-limit (4.30265) are considered significant

Optimization of the significant factors affecting electroporation efficiency

The top four factors from the Plackett–Burman design—DL-threonine, Tween 80, glycine in the weakening solution, and voltage during electroporation—were selected for subsequent optimization experiments, while all other factors were held constant. Firstly, the steepest uphill ascent and step size were determined based on the magnitude of the effect of the significant factors (Additional file 2: Table S5). The electroporation efficiency of GLB191 initially increases and then decreases with the increase of the concentration of DL-threonine, Tween 80, and glycine in the weakening solution, as well as with the decrease in the voltage used during electroporation. Among the tested combinations, run 2 (0.40 M Glycine, 0.20 M DL-threonine, 0.02% Tween 80, and 2.20 kV using a 2 mm electroporation cuvette) exhibited the highest transformation efficiency. Therefore, run 2 was used as the central configuration for the subsequent Box–Behnken experimental design to determine the interactions of each factor on electroporation efficiency.

A Box–Behnken experimental design was employed to assess the interactions of factors [glycine (*A*), DL-threonine (*B*), and tween 80 (*C*) in the weakening solution, and the voltage (*D*) during electroporation] influencing electroporation efficiency (Box and Behnken 1960). Three levels of each of the factors were used in the assessment, resulting in a total of 29 experimental runs (Additional file 2: Table S6 and Table S7). The data obtained from the Box–Behnken-designed experiments was subjected to multivariate regression analysis (Table 3). The *F*-value of the resulting model was 13.87, with a *P*-value less than 0.0001. The lack-of-fit *F*-value was 1.29, with a *P*-value of 0.433 ($p > 0.05$), indicating that the regression model adequately fits the data and was suitable for use in subsequent electroporation optimization experiments. The *P*-values for the main effects *A* and *C* were 0.0493 and 0.0411, respectively, indicating that they were significant variables. Notably, the *P*-values for the main effects *B* and *D* were < 0.0001 and < 0.0007, respectively, indicating that they were highly significant. Furthermore, the *P*-values for the quadratic terms A^2, B^2, and D^2 were 0.0008, 0.0004, and less than 0.0001, respectively, which

Table 3 ANOVA of the regression model obtained from the Box–Behnken experimental design data conducted to assess the impact of significant factors on the electroporation efficiency of GLB191

Source	Sum of squares	df	Mean square	F-value	*P*-value	Significance[a]
Model	4781.43	14	341.53	13.87	< 0.0001	**
A (glycine)	114.08	1	114.08	4.63	0.0493	*
B (DL-threonine)	1833.56	1	1833.56	74.46	< 0.0001	**
C (Tween 80)	124.59	1	124.59	5.06	0.0411	*
D (voltage)	464.59	1	464.59	18.87	0.0007	**
AB	2.25	1	2.25	0.0914	0.7669	
AC	0.1111	1	0.1111	0.0045	0.9474	
AD	13.44	1	13.44	0.5459	0.4722	
BC	21.78	1	21.78	0.8843	0.363	
BD	25	1	25	1.02	0.3308	
CD	0.1111	1	0.1111	0.0045	0.9474	
A^2	442.08	1	442.08	17.95	0.0008	**
B^2	516.36	1	516.36	20.97	0.0004	**
C^2	77.83	1	77.83	3.16	0.0972	
D^2	1830.14	1	1830.14	74.32	< 0.0001	**
Residual	344.76	14	24.63			
Lack of fit	263.34	10	26.33	1.29	0.433	
Pure error	81.42	4	20.36			
Cor total	5126.2	28				

[a] * and ** indicate statistical significance at $p < 0.05$ and $p < 0.01$, respectively

were also highly significant. These findings underscore the strong effect that the concentration of DL-threonine, Tween 80, glycine in the weakening solution, and the voltage employed during electroporation have on the electroporation efficiency of GLB191. The ranking of the magnitude of their effect, from large to small, are as follows: DL-threonine (F-value = 74.46), voltage during electroporation (F-value = 18.87), Tween 80 (F-value = 5.06), and glycine (F-value = 4.63). Regression analysis yielded a second-degree polynomial equation to estimate electroporation efficiency in relation to DL-threonine, Tween 80, and glycine in the weakening solution, and the voltage during electroporation:

$$\begin{aligned} Y = {} & 69.4 + 3.08333 \times A + 12.3611 \times B + 3.22222 \times C \\ & + 6.22222 \times D - 0.75 \times AB - 0.166667 \times AC \\ & - 1.83333 \times AD + 2.33333 \\ & \times BC + 2.5 \times BD - 0.166667 \times CD - 8.25556 \times A^2 \\ & - 8.92222 \times B^2 - 3.46389 \times C^2 - 16.7972 \times D^2. \end{aligned}$$

The model produced a multiple correlation coefficient R^2 of 0.9327, and an adjusted R^2 of 0.8655, indicating that the model accounted for 86.55% of the variation in electroporation efficiency response. The three-dimensional response surface plots and contour plots depicting the interactive effects of the factors on electroporation efficiency are presented in Fig. 2. The steeper the slope and the denser the contour lines, the more significant the impact. When the contour lines approximate an elliptical shape, the interaction between the two factors becomes stronger. Within the experimental range, all factors exhibit an interactive influence on the efficiency of electroporation.

Validation of electroporation efficiency

The highest transformation efficiency of 76.54 CFU/μg DNA was attained when the glycine concentration in the weakening solution was 0.42 M, DL-threonine concentration was 0.28 M, Tween 80 concentration was 0.03% (v/v), and the voltage during electroporation was 11.24 kV/cm (using a 2 mm electroporation cuvette). Subsequent experiments conducted under the optimized conditions validated the model, providing a transformation efficiency of 74.00 ± 5.10 CFU/μg of DNA, which was not significantly different ($p < 0.05$) from the efficiency predicted by the model (Table 4).

The effect of host restriction-modification systems on transformation efficiency

E. coli strains with various defective restriction-modification systems have been used for plasmid amplification to investigate the effect of host restriction-modification systems on the transformation efficiency of xenogeneic plasmids (Zhang et al. 2012). We utilized the

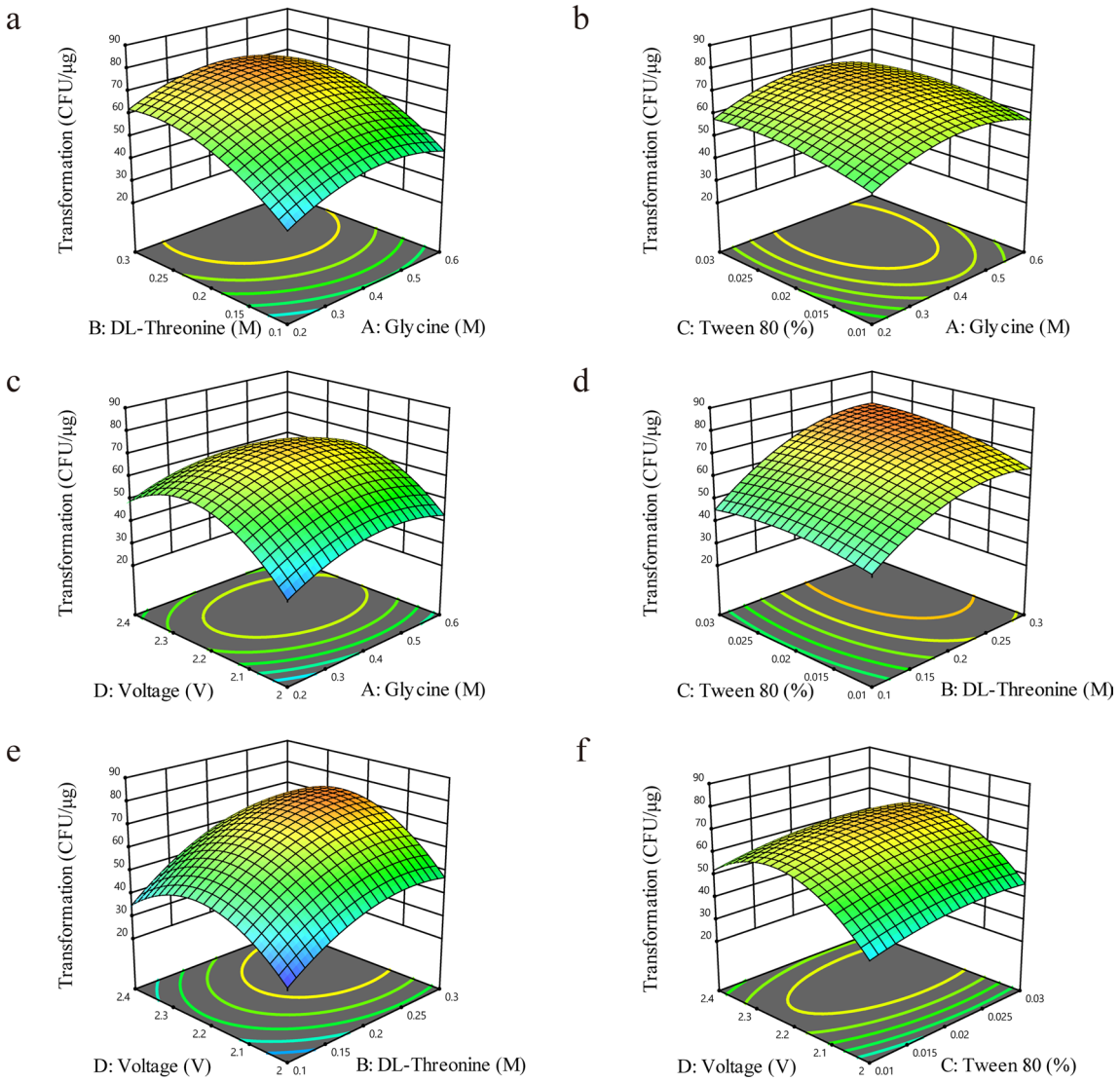

Fig. 2 Three-dimensional surface and two-dimensional contour plots of the impact of different factors on electroporation efficiency. When investigating the correlation between two factors and electroporation efficiency, the actual values of both external factors are individually set to 0 at the coded level. **a**–**f** respectively represent the interactions between two of the four factors (A-D) influencing electroporation efficiency

optimized electroporation conditions to introduce the plasmids into GLB191. *E. coli* DH5α, deficient in the *hsdR*-mediated restriction-modification system, exhibited a transformation efficiency of 3.00 ± 2.16 CFU/μg DNA for pDG1662 and 70.00 ± 0.82 CFU/μg DNA for pHT01 (Table 5). *E. coli* TOP10, which lacks all known *E. coli* restriction-modification systems, exhibited a transformation efficiency of 3.00 ± 2.45 CFU/μg DNA for pDG1662 and 58.33 ± 6.55 CFU/μg DNA for pHT01. In addition to having all known *E. coli* restriction-modification systems eliminated, *E. coli* HST04 is also missing the orphan methyltransferases *dam* and *dcm*. The transformation efficiency in *E. coli* HST04 was 11.33 ± 1.25 CFU/μg DNA for pDG1662 and $1.96 \pm 0.05 \times 10^6$ CFU/μg DNA for pHT01. The transformation efficiency for plasmids propagated in *E. coli* DH5α and *E. coli* TOP10 were not significantly different, while both were extremely significantly lower than the transformation efficiency of plasmids propagated in the *E. coli* HST04 strain, indicating that the electroporation efficiency of GLB191 was significantly enhanced due to the absence of *dam* and *dcm* modifications of the plasmid. However, when plasmids propagated in the *E. coli* HST04 strain were electroporated under nonoptimized conditions, the transformation efficiency for pDG1662 was 7.33 ± 3.09 CFU/μg DNA

Table 4 Optimized conditions to obtain maximum transformation efficiency

Glycine (M)	DLthreonine (M)	Tween 80 (%)	Voltage (KV)	Predicted transformation efficiency (CFU/μg of DNA)	Desirability	Experimental transformation efficiency (CFU/μg of DNA)
0.42	0.28	0.03	2.25	76.54	0.93	74.00 ± 5.10

Different letters in the same row indicate significant differences between different treatments according to the least significant difference (LSD) test ($p < 0.05$)

Table 5 Effect of host restriction-modification systems on transformation efficiency (CFU/μg of DNA)

DNA source	Optimized			Unoptimized
	E. coli DH5α	*E. coli* TOP10	*E. coli* HST04	*E. coli* HST04
Integrative plasmid pDG1662	3.00 ± 2.16 b	3.00 ± 2.45 b	11.33 ± 1.25 a	7.33 ± 3.09 b
Replicative plasmid pHT01	70.00 ± 0.82 c	58.33 ± 6.55 c	$1.96 \pm 0.05 \times 10^6$ a	120.33 ± 22.07 b

Different letters in the same row indicate significant differences between different treatments according to the least significant difference (LSD) test ($p < 0.05$)

and 120.33 ± 22.07 CFU/μg DNA for pHT01 (Table 5). Although higher than the transformation efficiency of plasmids propagated in *E. coli* DH5α and *E. coli* TOP10 strains, it was still significantly lower than the transformation efficiency obtained using the optimized electroporation conditions for *E. coli* HST04. Thus, the host restriction-modification system in GLB191 had a significant impact on transformation efficiency, and using the RSM-optimized electroporation protocol developed in our study with plasmids propagated in *E. coli* HST04 significantly enhanced transformation efficiency.

High electroporation efficiency facilitates effective genetic manipulation

To confirm the utility of the optimized electroporation strategy developed in our study, we conducted markerless knockout experiments on GLB191 using the pJOE8899 plasmid. The pJOE8899 plasmid, which possesses the pE194ts replicon from *Staphylococcus aureus*, is compatible with *B. subtilis* and is temperature-sensitive, ceasing replication at temperatures > 37 °C (Altenbuchner 2016). We generated a markerless deletion of the c-di-GMP phosphodiesterase gene *pdeH*, by integrating upstream and downstream homologous arms of *pdeH* into the pJOE8899 plasmid, resulting in the recombinant plasmid pJOE-*pdeH* (Fig. 3a). We introduced the recombinant plasmid pJOE-*pdeH* into GLB191 using the optimized electroporation strategy and achieved an electroporation efficiency of 2.1×10^6 CFU/μg DNA. The first homologous recombination strain was screened at 50 °C, while the second homologous recombination was screened at 28 °C (Fig. 3a). Sixteen randomly selected positive clones were verified. Genomic DNA of the resulting bacterial clones was amplified by PCR using P3/P6 primers, and the band of the expected size was sequenced to confirm the successful deletion of the *pdeH* gene, demonstrating a 100% gene knockout efficiency (Fig. 3b). PdeH, a key c-di-GMP phosphodiesterase in *B. subtilis*, has a critical function and its deletion results in the accumulation of c-di-GMP (Chen et al. 2012; Gao et al. 2013). The Δ*pdeH* mutation impairs the motility of GLB191 (Fig. 3c), which is consistent with previous reports (Chen et al. 2012; Gao et al. 2013). The results indicate that the optimized electroporation strategy facilitates effective genetic manipulation.

Discussion

In this study, the transformation efficiency of natural transformation and electroporation methods was compared, and an electroporation protocol for transforming nondomesticated *B. subtilis* strains was systematically optimized using response surface methodology. Factors such as growth medium during competent cell preparation, cell wall-weakening agents, electroporation buffer, and host restriction-modification system response to different modified plasmids were optimized to achieve maximum electroporation efficiency. The optimized electroporation protocol is presented in Additional file 2: Table S8.

Bacterial transformation experiments are commonly based on single-factor experimental manipulations, overlooking potential interactions between one or more additional factors. However, the existing literature has documented the major impact of the interactions that occur between growth media, recovery media, electric field strength, and various physical parameters, on transformation efficiency (Marciset and Mollet 1994). RSM is a multivariate experimental design tool that optimizes response variables by exploring relationships between multiple factors (Box and Wilson 1992). Box–Behnken is a type of response surface method based on a three-level and second-order experimental design protocol, using

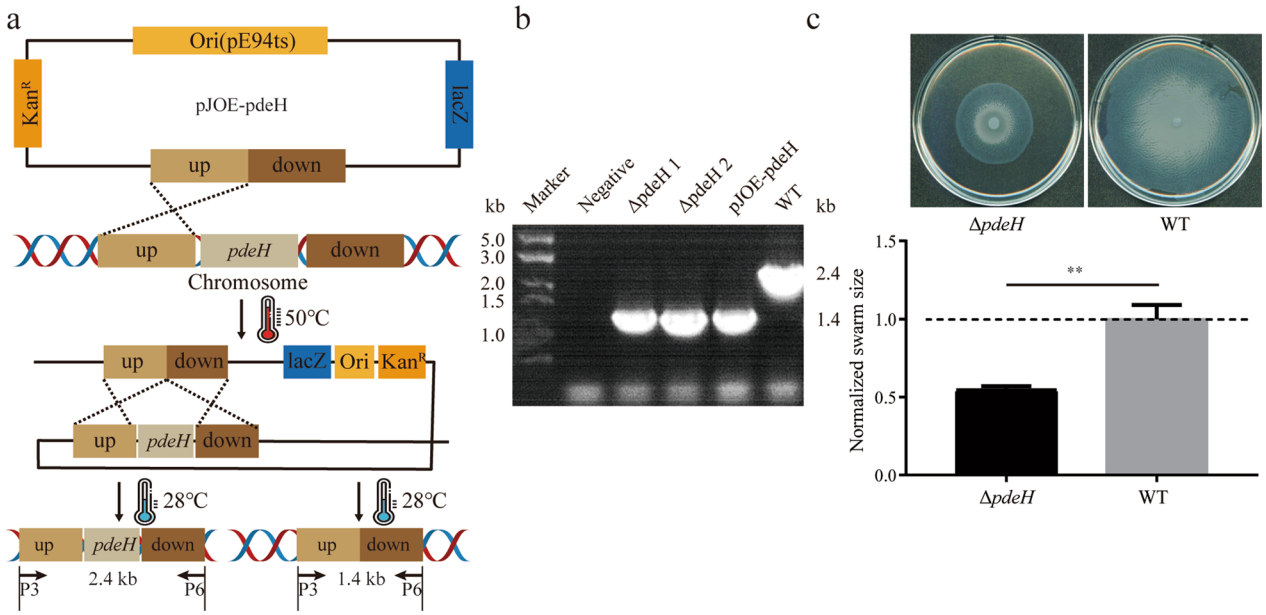

Fig. 3 Markerless genetic manipulation in *B. subtilis* GLB191. **a** Schematic representation of the markerless genome editing process using homologous recombination. The temperature shown beside the arrows indicates the incubation temperature required for each screening stage. P3 and P6 represent the primers used in the PCR validation of gene deletion. **b** PCR verification of successful generation of the *pdeH* knockout, with Δ*pdeH* 1–2 representing randomly selected clones, pJOE-pdeH and WT indicating positive controls for prospective mutants and the wild-type, respectively. **c** Swarming motility assay conducted on both wild-type and Δ*pdeH* strains. Graph indicates mean normalized swarm size ± SE ($n = 3$). ** denote significant differences with *t*-test, $p \leq 0.01$

multiple quadratic equations to fit factors, that can allow to determine the optimal value of a response variable under different experimental conditions (Ferreira et al. 2007; Bezerra et al. 2008). In the present study, a Plackett-Burman experimental design was employed to determine key factors affecting electroporation efficiency (Table 2; Fig. 1). Following that, a steepest ascent experiment was conducted to determine the center values of these factors (Additional file 2: Table S5). Then, a Box–Behnken experimental design, incorporating linear, interactive, and quadratic effects related to weakening agents and electric field intensity, was utilized to optimize the electroporation protocol (Table 3; Fig. 2). The resulting data was used to establish an optimal electroporation protocol based on a multivariate regression model that incorporated quadratic terms. Experiments were then conducted to validate the model and the experimental results exhibited a high level of agreement with the model predictions (Table 4).

Weakening agents are substances used to facilitate the preparation of competent cells in gram-positive bacteria for use in electroporation. This is accomplished by loosening the cell wall and altering the integrity of the cell membrane. Glycine and DL-threonine have the ability to penetrate bacterial cell walls and reduce the level of cross-linking of peptidoglycan layers, thereby enhancing the contact between the cell wall and exogenous DNA (McDonald et al. 1995). Studies have demonstrated that high concentrations of glycine or DL-threonine significantly increase the transformation efficiency in several gram-positive bacteria, including *B. cereus*, *Lactococcus lactis*, and *Corynebacterium glutamicum* (Holo and Nes 1989; Liebl et al. 1989; Peng et al. 2009). The magnitude of the electric field applied during electroporation also exerts a substantial influence on transformation efficiency. Higher electric field strengths within a specific range enhance the likelihood of exogenous DNA entering cells. Notably, elevating the electric field strength also leads to an increase in the level of cell mortality. In our study, glycine, DL-threonine, Tween 80, and electric field strength were identified as the main factors impacting the electroporation efficiency of GLB191, which is probably due to their effect on cell wall and cell membrane integrity.

Generally, the efficiency of bacterial transformation utilizing DNA from non-native species is often considerably lower compared to the use of homologous DNA due to the higher susceptibility of foreign DNA to host restriction systems. In this regard, *E. coli* is commonly used as the host bacterium for the construction of plasmid DNA for subsequent use in genome editing. Restriction-modification systems present in *Bacillus*, however,

can degrade foreign DNA, resulting in low transformation efficiency when plasmids propagated in *E. coli* are utilized. Thus, there is a definite need to overcome the restriction barriers associated with intergeneric DNA transfer between *E. coli* and *Bacillus*. In the present study, a substantial disparity in transformation efficiency was observed when plasmids propagated in specific modified strains of *E. coli* were used. The use of the *E. coli* HST04 strain, which lacks all *E. coli* restriction-modification systems and orphan methyltransferases, resulted in a six-order of magnitude improvement in transformation efficiency (Table 5). This result suggests the presence of a thus far unreported Type IV restriction-modification system recognizing methyltransferases *dam* and *dcm* in GLB191, which necessitates further study.

Conclusions

A detailed electroporation protocol was developed in the present study that optimizes the electroporation efficiency of nondomesticated *B. subtilis* GLB191. The highest electroporation efficiency achieved for the xenogeneic pHT01 shuttle plasmid using this strategy was $1.96 \pm 0.05 \times 10^6$ CFU/μg DNA. Efficient genetic transformation enables researchers to explore the genetic manipulation of *B. subltilis* to conduct basic molecular and biochemical studies.

Methods

Bacterial strains, plasmids, and media

The bacterial strains and plasmids employed in this study are presented in Additional file 2: Table S9. *E. coli* and *B. subtilis* strains were regularly cultured in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) or on LB plates containing 1.5% (w/v) agar at 37 °C and 30 °C, respectively. The 10 × S-base (Spizizen's salt) used to prepare HS medium and LS medium for natural competence of *B. subtilis* was as follows: 2 g/L $(NH_4)_2SO_4$, 14 g/L K_2HPO_4, 6 g/L KH_2PO_4, 1 g/L sodium citrate, 12 mg/L $MgSO_4$. The recipe of HS medium is as follows: Per 100 mL contain 66.5 mL H_2O, 10 mL 10 × S-base, 2.5 mL 20% [w/v] glucose, 5 mL 0.1% [w/v] L-tryptophan, 1 mL 2% [w/v] casamino acids, 5 mL 10% [w/v] yeast extract, 10 mL 8% [w/v] arginine, and 0.4% [w/v] histidine. The recipe of LS medium is as follows: Per 98.8 mL contain 80 mL H_2O, 10 mL 10 × S-base, 2.5 mL 20% [w/v] glucose, 0.5 mL 0.1% [w/v] L-tryptophan, 0.5 mL 2% [w/v] casamino acids, 5 mL 2% [w/v] yeast extract, 0.25 mL 1 M $MgCl_2$, 0.05 mL 1 M $CaCl_2$. *B. subtilis* cultures were maintained at 50 °C during plasmid curing and at 28℃ during counter-selection of genome editing procedures. When necessary, the medium was supplemented with ampicillin (100 μg/mL for *E. coli*), chloramphenicol (5 μg/mL for *Bacillus*), tetracycline (15 μg/mL for *Bacillus*), kanamycin (50 μg/mL and 20 μg/mL for *E. coli* and *Bacillus*, respectively).

Construction of recombinant plasmids

Primers utilized in this study are listed in Additional file 2: Table S10. For the construction of pJOE-*pdeH*, the pJOE8899 plasmid was subjected to double digestion with *Afl* II and *Eco*R I enzymes to yield pMB1-KanR-Ori sequence fragments. Subsequently, the *lacZ* sequence fragment was PCR-amplified utilizing primers P1 and P2 and the *E. coli* pHY306-lacZ plasmid as the template. The upstream and downstream homologous arms of the *pdeH* sequence fragment were PCR-amplified using P3/P4 and P5/P6. All fragments were then isolated using an agarose gel DNA extraction kit (Takara Technologies Inc, Japan). The recombinant plasmid pJOE-*pdeH* was assembled by combining all fragments using a seamless assembly cloning kit (Clone Smarter Technologies Inc, USA) and introduced into *E. coli* DH5α. Positive clones were identified as colonies displaying growth on LB plates supplemented with 50 μg/mL of kanamycin.

Natural transformation

The procedure employed in this study for the natural competence of *B. subtilis* was adopted from Klein et al. (1992) and as follows: a single fresh colony from an LB plate was inoculated in 5 mL of HS medium and incubated overnight at 37℃. The culture was then transferred to 50 mL of fresh HS medium and incubated until an OD_{600} of 0.05 was obtained. The culture was continuously shaken at 37℃, and OD_{600} values were recorded to generate a growth curve. Once the culture reached the steady growth phase after the transition from exponential growth, a 10 mL sample was immediately taken and mixed with 1 mL of sterile 87% glycerol to prepare competent cells. For transformation, 1 mL of competent cells was inoculated into 20 mL of LS medium and incubated at 30℃ for 2 h. Subsequently, 1 mL of the culture was transferred to a 2 mL sterile centrifuge tube and mixed with 10 μL of 0.1 M EGTA, followed by incubation at room temperature for 5 min. Then, 1 μg of DNA was added, and the culture was further incubated at 37℃ for 2 h (Klein et al. 1992).

The procedure used for transformation with *comK* and *comS* overexpression was as follows: a single fresh colony from an LB plate was inoculated into 3 mL of LB broth and incubated overnight at 37℃ on a rotary shaker. Then, 2 mL of fresh LB medium supplemented with 2.5% (w/v) mannitol and xylose was added, and the culture was further incubated for 2 h to induce the formation of competent cells. DNA was then added directly to the culture to

conduct the transformation, and the transformation culture was incubated for 90 min (Deng et al. 2021).

The resulting transformed mixture was plated onto LB agar plates supplemented with the appropriate selection antibiotics, followed by overnight incubation to determine transformation efficiency, measured as the number of transformants obtained per microgram of plasmid DNA.

Electroporation

A single fresh colony from an LB plate was inoculated into 5 mL of LB medium and incubated overnight at 37 °C. The overnight culture was then transferred to 100 mL of fresh growth medium (LB medium containing sorbitol) at a 1% (v/v) ratio and incubated at 37 °C on a rotary shaker until reaching an OD_{600} of 0.6–1. The cell wall was weakened, and cell membrane integrity was disrupted by the addition of compounds including mannitol, DL-threonine, and Tween 80 to the culture, and shaking for 10 min. The culture was then cooled on ice for 20 min, after which cells were collected by centrifugation at 3466 *g* at 4 °C for 10 min. The cells were then washed with ice-cold electroporation buffer and resuspended in 1 mL to prepare electro-competent cells.

For transformation using electroporation, 100 μL of electro-competent cells were mixed with 100 ng of DNA and incubated on ice for 20 min. The cell-DNA mixture was then transferred to a pre-chilled, 0.2 cm electroporation cuvette and subjected to electroporation using an electroporator (Bio-RAD, USA). Following electroporation, 1 mL of 42 °C preheated recovery medium (LB medium containing mannitol) was immediately added to the mixture. The putative transformed cells were then transferred to 12 mL sterile round-bottom culture tubes and placed at 37 °C on a rotary shaker set at 150 rpm for 3 h to facilitate recovery. Lastly, the transformation mixtures were evenly spread on LB agar plates supplemented with the necessary antibiotics.

Optimization of electroporation efficiency using response surface methodology

A Plackett-Burman experimental design was used to identify the factors with a significant impact on electroporation efficiency (Plackett and Burman 1946). Subsequently, a steepest ascent experiment was conducted to determine the center values for the Box–Behnken design. The Box–Behnken model utilizes an equation with quadratic terms for multiple experimental factors to evaluate the relationship between experimental factors and responses at three levels (− 1, 0, 1). The required number of experiments that need to be conducted for the Box–Behnken experimental design can be calculated using the following formula (Eq. 1) (Ferreira et al. 2007).

$$N = 2k(k-1) + C_0 \quad (1)$$

where N represents the number of runs needed in the Box–Behnken experimental design, k denotes the number of experimental factors, and C_0 corresponds to the number of central points (Ferreira et al. 2007).

Genome editing

Genome editing involves two main steps: (I) screening for the first homologous recombination and plasmid removal, and (II) counter-selection for the second homologous recombination and validation of positive clones. The recombinant plasmid pJOE-*pdeH* was initially transformed into *B. subtilis* using the optimized electroporation method developed in the present study. The transformed cells were then spread onto LB agar plates supplemented with 20 μg/mL of kanamycin and incubated overnight at 28℃. Single colonies were selected and transferred to liquid LB medium, followed by incubation at 50℃ on a rotary shaker set at 200 rpm until the optical density (OD_{600}) reached between 0.6 and 1. Subsequently, a gradient dilution was performed, and the diluted samples were spread onto LB agar plates containing kanamycin. These plates were incubated at 50℃ for 6 h. During this incubation period, the recombinant plasmid undergoes homologous recombination with the target gene, leading to the segregation of the nonintegrated vector. This occurred when cells were cultured on LB plates containing kanamycin at the non-permissive temperature of 50℃. The successful occurrence of the first homologous recombination, known as the single exchange clone, was confirmed using blue-white selection (LB medium supplemented with 40 μg/mL X-gal).

Counter-screening and validation of positive clones were performed to select clones that underwent a second round of homologous recombination, resulting in the intended genome modification. One single exchange clone was selected and transferred to an antibiotic-free LB medium. The culture was then incubated overnight at 28℃ on a rotary shaker set at 200 rpm. Gradient dilution was performed following this incubation period and the dilutions were plated onto LB agar plates. The plates were then incubated until visible colonies were observed. White colonies represent successfully double-exchanged clones. Lastly, PCR was employed to confirm the mutant strains.

Swarming motility assay

The experimental procedure described by Chen et al. (2012) with some modifications was used to assess the swarming motility of *B. subtilis*. A fresh colony was initially selected from an LB agar plate and inoculated into 3 mL of LB medium. The culture was then incubated at 37 °C on a rotary shaker until reaching an optical density of 1 (OD_{600}). The bacterial cells were then harvested by centrifugation at 3466 *g* for 5 min at room temperature and then washed by resuspending them in 1 mL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4). The bacterial cells were subsequently resuspended in 100 µL of PBS. LB agar plates (0.7% w/v) were prepared and allowed to dry in a laminar flow cabinet for 10 min. Then, 5 µL of the bacterial suspension was inoculated in the center of each plate, after which the plates were left to dry for an additional 5 min. The swarming experiment was conducted at 37 °C. Once the control group of bacteria reached the periphery of the culture dish, all plates were air-dried for 1 h in a laminar flow cabinet to halt bacterial motility. Finally, the plates were incubated at 37 °C until visible swarming tracks were observed, which were then photographed.

Processing of data

The experimental design was established and an ANOVA for the response surface methodology was conducted using Design-Expert software with default parameters. This software automatically generates 3D response surface graphs and 2D contour plots.

Abbreviations

LB	Luria–Bertani
CFU	Colony Forming Units
ANOVA	Analysis of variance
X-gal	5-Bromo-4-chloro-3-indolyl β-d-galactopyranoside

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00287-0.

Additional file 1: Figure S1. Increased expression of comK and comS improves natural competence of GLB191.

Additional file 2: Table S1. Plasmid curing efficiency in GLB191. **Table S2.** Selected factors used in the Placket–Burman experimental design. **Table S3.** Plackett–Burman experimental design and resulting response values. **Table S4.** Factors influencing electroporation and their effects. **Table S5.** Steepest ascent experimental design and results. **Table S6.** Box–Behnken experimental design used to determine the impact of significant factors on the electroporation efficiency of GLB191. **Table S7.** Response values obtained utilizing the Box–Behnken experimental design. **Table S8.** Optimal electroporation conditions determined in the present study. **Table S9.** Plasmids and strains utilized in the present study. **Table S10.** Primer sequences employed in the present study.

Acknowledgements

We thank Prof. Tingyi Wen (Key Laboratory of Systems Microbial Biotechnology, Institute of Microbiology, Chinese Academy of Sciences) for kindly providing the pWYB108 plasmid.

Author contributions

YL initiated, coordinated, and supervised the project. YZ and QW conducted the experiments and gathered the data. YZ analyzed the data and drafted the manuscript. ZW, QZ, QW, and YL reviewed and revised the manuscript. All authors read and approved the final version of the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (32272613, 31972982), the National Key R&D Program of China (2023YFE0104800), and the Science and Technology Commissioner Industry University Research Cooperation Project of Sanming City (No. 2022-N-18).

Data availability

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 14 March 2024 Accepted: 11 November 2024
Published online: 12 December 2024

References

Altenbuchner J. Editing of the *Bacillus subtilis* genome by the CRISPR-Cas9 system. Appl Environ Microbiol. 2016;82:5421–7.
Berg G. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl Microbiol Biotechnol. 2009;84:11–8.
Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta. 2008;76:965–77.
Box GE, Behnken DW. Some new three level designs for the study of quantitative variables. Technometrics. 1960;2:455–75.
Box GE, Wilson KB. On the experimental attainment of optimum conditions. In: Breakthroughs statistics: methodol distribution. 1992:270–310.
Chang S, Cohen SN. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol Gen Genet. 1979;168:111–5.
Chen Y, Chai Y, Guo JH, Losick R. Evidence for cyclic di-GMP-mediated signaling in *Bacillus subtilis*. J Bacteriol. 2012;194:5080–90.
Deng A, Sun Z, Wang T, Cui D, Li L, Liu S, et al. Simultaneous multiplex genome engineering via accelerated natural transformation in *Bacillus subtilis*. Front Microbiol. 2021;12:714449.
Dong H, Zhang D. Current development in genetic engineering strategies of *Bacillus* species. Microb Cell Fact. 2014;13:63.
Ferreira SL, Bruns RE, Ferreira HS, Matos GD, David JM, Brandao GC, et al. Box-Behnken design: an alternative for the optimization of analytical methods. Anal Chim Acta. 2007;597:179–86.
Gao X, Mukherjee S, Matthews PM, Hammad LA, Kearns DB, Dann CE IIIrd. Functional characterization of core components of the *Bacillus subtilis* cyclic-di-GMP signaling pathway. J Bacteriol. 2013;195:4782–92.
Holo H, Nes IF. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl Environ Microbiol. 1989;55:3119–23.

Jarmer H, Berka R, Knudsen S, Saxild HH. Transcriptome analysis documents induced competence of *Bacillus subtilis* during nitrogen limiting conditions. FEMS Microbiol Lett. 2002;206:197–200.
Jeong DE, Kim MS, Kim HR, Choi SK. Cell factory engineering of undomesticated *Bacillus* strains using a modified integrative and conjugative element for efficient plasmid delivery. Front Microbiol. 2022;13:802040.
Klein C, Kaletta C, Schnell N, Entian KD. Analysis of genes involved in biosynthesis of the *lantibiotic subtilin*. Appl Environ Microbiol. 1992;58:132–42.
Liebl W, Bayerl A, Schein B, Stillner U, Schleifer KH. High efficiency electroporation of intact *Corynebacterium glutamicum* cells. FEMS Microbiol Lett. 1989; 65.
Lu YP, Zhang C, Lv FX, Bie XM, Lu ZX. Study on the electro-transformation conditions of improving transformation efficiency for *Bacillus subtilis*. Lett Appl Microbiol. 2012;55:9–14.
Mahipant G, Kato J, Kataoka N, Vangnai AS. An alternative genome-integrated method for undomesticated *Bacillus subtilis* and related species. J Gen Appl Microbiol. 2019;65:96–105.
Marciset O, Mollet B. Multifactorial experimental design for optimizing transformation: electroporation of *Streptococcus thermophilus*. Biotechnol Bioeng. 1994.
McDonald IR, Riley PW, Sharp RJ, McCarthy AJ. Factors affecting the electroporation of *Bacillus subtilis*. J Appl Bacteriol. 1995;79:213–8.
Peng D, Luo Y, Guo S, Zeng H, Ju S, Yu Z, et al. Elaboration of an electroporation protocol for large plasmids and wild-type strains of *Bacillus thuringiensis*. J Appl Microbiol. 2009;106:1849–58.
Plackett RL, Burman JP. The design of optimum multifactorial experiments. Biometrika. 1946;33:305–25.
Roberts RJ, Belfort M, Bestor T, Bhagwat AS, Bickle TA, Bitinaite J, et al. A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. Nucleic Acids Res. 2003;31:1805–12.
Roberts RJ, Vincze T, Posfai J, Macelis D. REBASE: a database for DNA restriction and modification: enzymes, genes and genomes. Nucleic Acids Res. 2022;51:D629–30.
Schallmey M, Singh A, Ward OP. Developments in the use of *Bacillus* species for industrial production. Can J Microbiol. 2004;50:1–17.
She Q, Hunter E, Qin Y, Nicolau S, Zalis EA, Wang H et al. Negative interplay between biofilm formation and competence in the environmental strains of *Bacillus subtilis*. mSystems. 2020; 5.
Tran LS, Nagai T, Itoh Y. Divergent structure of the ComQXPA quorum-sensing components: molecular basis of strain-specific communication mechanism in *Bacillus subtilis*. Mol Microbiol. 2000;37:1159–71.
van der Rest ME, Lange C, Molenaar D. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl Microbiol Biotechnol. 1999;52:541–5.
Xue GP, Johnson JS, Dalrymple BP. High osmolarity improves the electrotransformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J Microbiol Methods. 1999;34:183–91.
Yasbin RE, Young FE. Transduction in *Bacillus subtilis* by bacteriophage SPP1. J Virol. 1974;14:1343–8.
Zhang GQ, Bao P, Zhang Y, Deng AH, Chen N, Wen TY. Enhancing electrotransformation competency of recalcitrant *Bacillus amyloliquefaciens* by combining cell-wall weakening and cell-membrane fluidity disturbing. Anal Biochem. 2011;409:130–7.
Zhang G, Wang W, Deng A, Sun Z, Zhang Y, Liang Y, et al. A mimicking-of-DNA-methylation-patterns pipeline for overcoming the restriction barrier of bacteria. PLoS Genet. 2012;8:e1002987.
Zhang X, Zhou Y, Li Y, Fu X, Wang Q. Screening and characterization of endophytic *Bacillus* for biocontrol of grapevine downy mildew. Crop Prot. 2017;96:173–9.