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BIOTECHNOLOGY METHODS - ORIGINAL PAPER

Construction of a plasmid interspecific transfer system in *Bacillus* species with the counter-selectable marker *mazF*

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Abstract

Bacillus sp. strains as attractive hosts for the production of heterologous secretory proteins usually play important roles in bio-industry. However, low transformation efficiency of exogenous plasmids limited the application of *Bacillus* species. Here, a novel plasmid interspecific transfer system, with high transformation efficiency, high positive rate, and convenient manipulation, has been successfully constructed. A high electrotransformation efficiency strain *Bacillus subtilis* F-168 containing the counter-selectable marker *mazF* was used as the plasmid donor strain in this transfer method. A shuttled plasmid pBE980 and its recombinant plasmids pBE980::*pulA* and pBE980::*HSPA* were successfully transferred into the recipient *Bacillus* strains (*Bacillus amyloliquefaciens* 66, *Bacillus licheniformis* 124 and *Bacillus megaterium* 258) by this method. After co-culturing the donor cells (OD_{600nm} = 1.3–1.7) and the recipient cells (OD_{600nm} = 0.5–0.9) for 24 h in 22 °C, more than 1.0×10^5 positive transformants were obtained and a interspecific transformation efficiency of 1.0×10^{-3}. It would provide a new approach for genetic manipulation in *Bacillus* strains and accelerate the research progress of the wild *Bacillus* strains in bio-industry.

Keywords Plasmid interspecific transformation · Counter-selectable marker · *Bacillus* species · *mazF*-cassette · *B. subtilis* 168 · pBE980

Introduction

Bacillus is a large genus of bacteria which shows typical phenotypes including Gram-positive, spore-forming, strict aerobic or facultative anaerobic [5, 10, 15]. Most *Bacillus* species are non-pathogenic and are capable of secreting functional extracellular proteins directly to the culture medium. Moreover, the mature large scale fermentation conditions of many *Bacillus* sp. strains have now been acquired. Because of these superiorities, *Bacillus* species are usually supposed to attractive hosts for the production of heterologous secretory proteins and play important roles in bio-industrial production [3, 13, 24]. In the near past years, with the development of molecular biology and genetic engineering, the genetic manipulation of *Bacillus* sp. strains has become the research focus [16, 25, 30].

Bacillus subtilis 168 as a model strain for molecular manipulation was first discovered to be transformable in 1958 [17]. Hereafter, plasmids with antibiotic markers, such as pE194 and pUB110, were isolated from *Staphylococcus aureus* and used as vectors in *B. subtilis* and other *Bacillus* strains [11]. Nowadays, a series of genetically engineered *Bacillus* strains (such as *Bacillus licheniformis*

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and *Bacillus megaterium*) and the corresponding plasmids were constructed successfully [8, 29]. However, efficiently transformation of exogenous plasmids is a critical factor to the development of this field [21]. At present, foreign plasmids are transferred into *Bacillus* strains mostly by electroporation, protoplast method and biochemical transformation. These three transformation ways are well-developed, but they still have some shortcomings [27]. Biochemical transformation has limited universality and is usually used in *B. subtilis* 168. Electroporation and protoplast method are applicable to a great variety of *Bacillus* species, but the complex manipulation and the difficulty for cell recovering limited their application. On the other hand, low transformation rates are normally found in these two methods [1, 4, 28]. According to previous reports, there are another two means of gene transfer except transformation: conjugation and transduction [18]. Based on the transfer mechanism of these two ways, some transfer agents such as bacteriophage or transposon are necessary. However, an aggregation-mediated plasmid transfer was found between two *Bacillus* strains without any transfer agents neither in the donor nor the recipient strains [27]. The universality and mechanism of this aggregation-mediated plasmid transfer need to be further studied. Therefore, it is still necessary to make efforts to construct an applicable, simple and efficient transfer system in *Bacillus* species. Especially, among the wild *Bacillus* strains, there are no efficient plasmid transfer methods to be reported so far.

In this study, a convenient and efficient plasmid interspecific transformation procedure was developed for the wild *Bacillus* strains. A shuttle plasmid pBE980 was successfully transferred from *B. subtilis* F-168 to the wild *Bacillus* strains *B. amyloliquefaciens* 66, *B. licheniformis* 124 and *B. megaterium* 258, respectively. And the donor strain (*B. subtilis* F-168) was constructed by integrating an IPTG inducible toxin-gene *mazF* cassette to the chromosome of *B. subtilis* 168. So, an efficiency counter-selectable method was proved to work for the selection of the positive recipient *Bacillus* strains. Therefore, this reported transformation procedure is more applicative to the wild *Bacillus* recipients with high transfer efficiency and application prospects.

Materials and methods

Bacterial strains, plasmids, oligonucleotides and materials

The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* 168 (ATCC23857) was provided

Table 1 Strains and plasmids used in this study

Strains and plasmids	Characteristics	Reference
Strains		
E. coli DH5α	F-, φ80d*lacZ*ΔM15, Δ (*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk–, mk+), *pho*A, *supE*44, λ-, *thi*-1, *gyrA*96, *relA*1	TaKaRa
B. subtilis 168[a]	Wild type	ATCC
B. subtilis F-168	168 derivative, *lacO*, *mazF*	This work
B. amyloliquefaciens 66[b]	Wild type, spec^{r}	This work
B. licheniformis 124	Wild type	This work
B. megaterium 258	Wild type	This work
Plasmids		
pHT43	Amp^{r} (*E. coli*), Chl^{r} (*B. subtilis*), *lacO*, *B. subtilis–E. coli* shuttle expression vector	MoBiTec
pHT43E	Amp^{r} (*E. coli*), Chl^{r} (*B. subtilis*), *B. subtilis–E. coli* shuttle expression vector, *lacO, mazE*	This work
pHT43EF	Amp^{r} (*E. coli*), Chl^{r} (*B. subtilis*), *B. subtilis–E. coli* shuttle expression vector, *lacO, mazE*, *mazF*	This work
pHT43EFD	Amp^{r} (*E. coli*), Chl^{r} (*B. subtilis*), *B. subtilis–E. coli* shuttle expression vector, *lacO, mazE*, *mazF*, *aprx*DOWN	This work
pHT43EUFD	Amp^{r} (*E. coli*), Chl^{r} (*B. subtilis*), *B. subtilis–E. coli* shuttle expression vector, *lacO, mazE*, *mazF*, *aprx*DOWN, *aprx*UP	This work
pUB110	Kan^{r}, *mob* region	Our lab. preservation
pWB980	pUB110 derivative, destroyed *mob* region, Kan^{r}, P43 promoter	Our lab. Preservation
pBE980	*B. subtilis–E. coli* shuttle expression vector, Kan^{r}, P43 promoter	This work
pBE980::*pulA*	*B. subtilis–E. coli* shuttle expression vector, Kan^{r}, P43 promoter, *pulA*	This work
pBE980::*HSPA*	*B. subtilis–E. coli* shuttle expression vector, Kan^{r}, P43 promoter, *HSPA*	This work

[a] *Bacillus subtilis* 168 (ATCC23857) was provided by the American Tissue Culture Collection (ATCC)

[b] *Bacillus amyloliquefaciens* 66 was deposited in the China General Microbiological Culture Collection Center (CGMCC14363)

by the American Tissue Culture Collection (ATCC). And the wild recipient strain *B. amyloliquefaciens* 66 was deposited in the China General Microbiological Culture Collection Center with the number CGMCC14363. The 16s rDNA sequences of *Bacillus amyloliquefaciens* 66, *Bacillus licheniformis* 124, and *Bacillus megaterium* 258 have been submitted to the GenBank database under accession No. MH150812, MH150817, and MH152576, respectively. The specific primers (Table S1) used for PCR amplification are synthesized by the Beijing Genomics Institute (BGI). The restriction endonucleases and DNA polymerase were commercially supplied by Thermo Fisher Scientific Co., Ltd. All other enzymes, chemicals and reagents were purchased from TaKaRa Biotechnology (Dalian, China) Co., Ltd.

Culture and growth conditions

All cells were routinely grown at 37 °C and 200 rpm in Luria–Bertani (LB) medium. LBG (LB containing 1% glucose) medium was used to prevent leaked expression of *mazF* gene in *B. subtilis* F-168. LB medium containing 0.8 mM IPTG was used to select transformants underlying the *mazF* counter-selectable system. Different antibiotics (100 μg/ml ampicillin, 25 μg/ml kanamycin, 150 μg/ml spectinomycin and 25 μg/ml chloramphenicol) were added in the medium for the relevant recombinant strains.

General molecular manipulations

The isolation and manipulation of recombinant DNA were performed using the methods as described by Sambrook and Russell [14]. The *E. coli* competent cells were obtained by regular way and transformed through chemical transformation method as described by Sambrook and Russell [14]. The electroporation transformation of *B. subtilis* 168 was carried out according to Tjalsma et al. [20].

Construction of the shuttle plasmid pBE980 and its recombinants

The *ori* gene from site 1694 to 2562 was amplified using primers ori-1/ori-2 (Table S1) from pUC19. Then the PCR products were inserted into pWB980 at the site between *Eco*RI and *Bg*lII which is on the upstream of P43 promoter (Fig. 1). The recombinant *E. coli–B. subtilis* shuttle expression vector was named as pBE980 in this work. Moreover, the recombinant vector pBE980::*pulA* and pBE980::*HSPA* were constructed by inserting a pullulanase encoding gene (*pulA*) and a protease encoding gene (*HSPA*) into the multiple cloning site (MCS) of pBE980, respectively. The *PulA* gene and *HSPA* gene were synthesized according to the sequences in GenBank database under HQ844266.1 and JX235368.1, respectively.

Relative plasmid copy number determination

The copy number of plasmids was determined by quantitative real-time PCR (qPCR) using iQ™ SYBR® Green Supermix (Bio-Rad). Primers were designed to have a predicted melting temperature of about 60 °C and to generate products of approximately 100 bp in length. Dilutions (10^{-1} to 10^{-4}) of total cellular DNA (10 μg) isolated from *B. subtilis* F-168 (containing test plasmids) were analyzed by qPCR using primer pairs specific for the *rep* gene. The copy numbers were calculated as the mean threshold cycle (C_T) values of the amplicons of the chromosomal genes (single-copy reference) compared to the amplicon of the plasmid-borne *rep* using the formula $2^{\Delta CT}$. ΔC_T is the difference between the threshold cycle number of the reference gene and that of the *rep* reaction [2].

Construction of *B. subtilis* F-168

Using *E. coli* chromosome DNA as the template, the *lpp* promoter and the *mazE* gene were amplified by PCR with primers P5/P6 and P7/P8 (Table S1), respectively. The two obtained fragments were then fused together by overlap PCR using primers P1/P2 (Table S1). After that, the PCR products were ligated to the sites between *Xho* I and *Bgl* II of pHT43 to form pHT43E. The *mazF* gene was amplified from the chromosome DNA of *E. coli* DH5α using the primers P9/P10 (Table S1), then inserted into the pHT43E to yield pHT43EF. With the primers P11/P12 and P13/P14 (Table S1), the upstream and downstream fragments of the *aprx* gene, *aprx*UP and *aprx*DOWN were amplified by PCR from *B. subtilis* 168 chromosome DNA and cloned into the corresponding site of pHT43EF resulting plasmid pHT43EUFD. When pHT43-EUFD was linearized by *Xho* I and then transformed into *B. subtilis* 168, a double crossover mutant strain was selected and named as *B. subtilis* F-168 (Fig. 2a).

The interspecific transformation

First, the target plasmids were transformed into *B. subtilis* F-168 using electroporation transformation method. The *B. subtilis* F-168 harboring exogenous plasmids was used as the plasmid donor. And the other *Bacillus sp*. strains were used as the plasmid recipients. The donor strain was cultured in LBG medium and the recipient strain was cultured in LB medium. They were separately cultured at 37 °C and 220 rpm for 10–12 h. And then 10% of each seed cultured broth was inoculated into the fresh LB medium for continuing cultivation. Until the donor cells grew to $OD_{600nm} = 1.3–1.8$ and the recipient cells grew to $OD_{600nm} = 0.5–1.0$, the cells were collected by centrifugating at 13,000*g* for 2 min. 10% of the supernatant was used

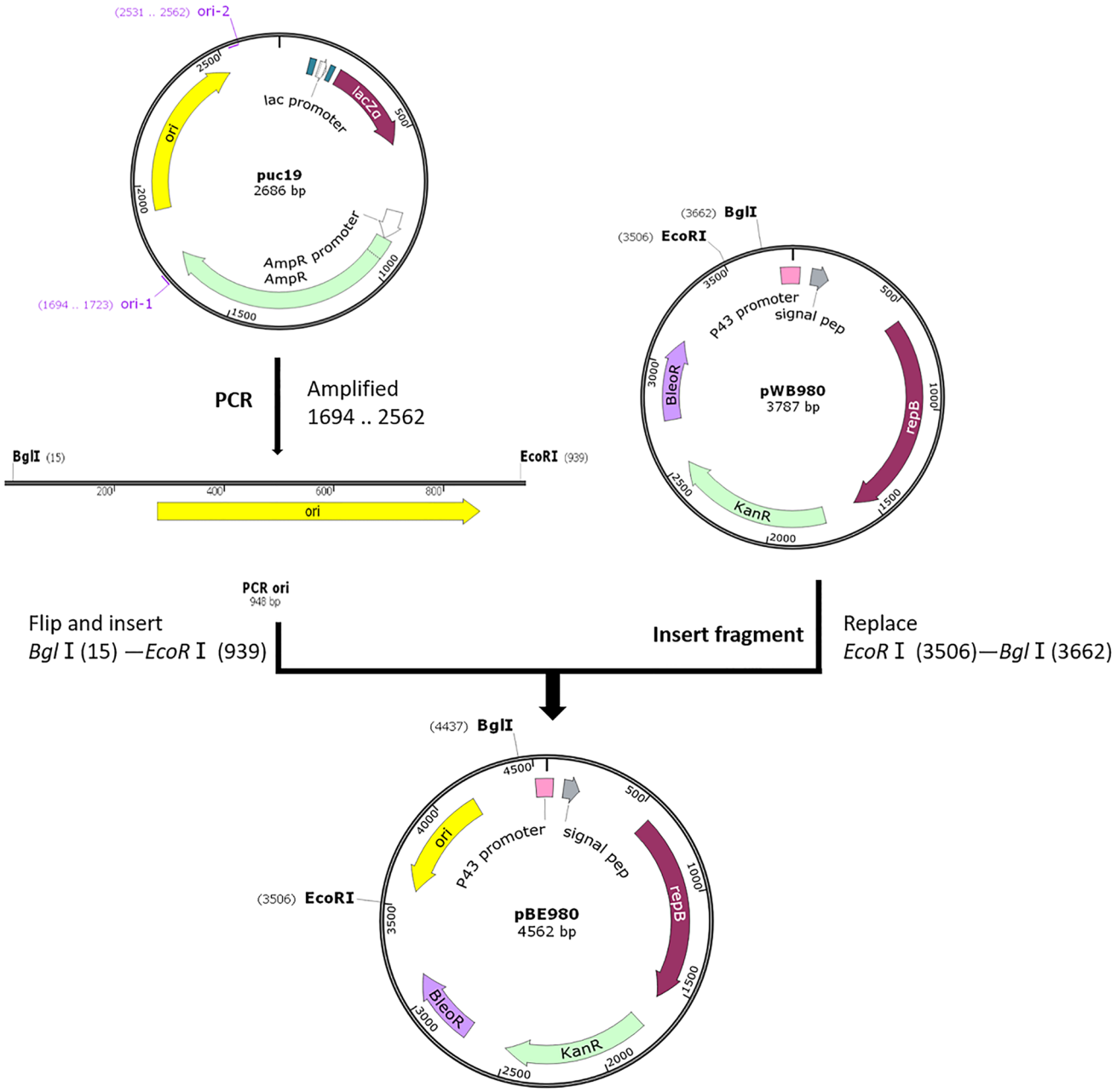

Fig. 1 The construction procedure of a novel *E. coli–B. subtilis* shuttle plasmid pBE980

to resuspend the cells. Immediately, the donor cells and recipient cells were mixed at a volume ratio of 3:7 into total 200 µl and co-incubated in 1.8 ml LB medium with 0.8 mM IPTG at 22 °C and 220 rpm for 24 h. As a control, 1 µg plasmid DNAs were mixed with the same volume of the recipient cells and co-incubated under the same condition. The positive recipient transformants were selected under the *mazF* counter-selectable system and confirmed by PCR or plasmid extraction.

The counter selection with *mazF*

After co-cultured of the donor cells and the recipient cells, the mixed cells (1 ml, $OD_{600nm} = 1.0$) with ten times dilution was spread on a LB plate with 0.8 mM IPTG. Then the plate was cultured at 37 °C for 24 h. The positive recipient transformants could be found through colonial morphology and there remained minimal residual donor cells on the plate.

$$\text{The lethallty rate of donor cells}(\%) = \frac{(T1 - R1)}{T1} \times 100,$$

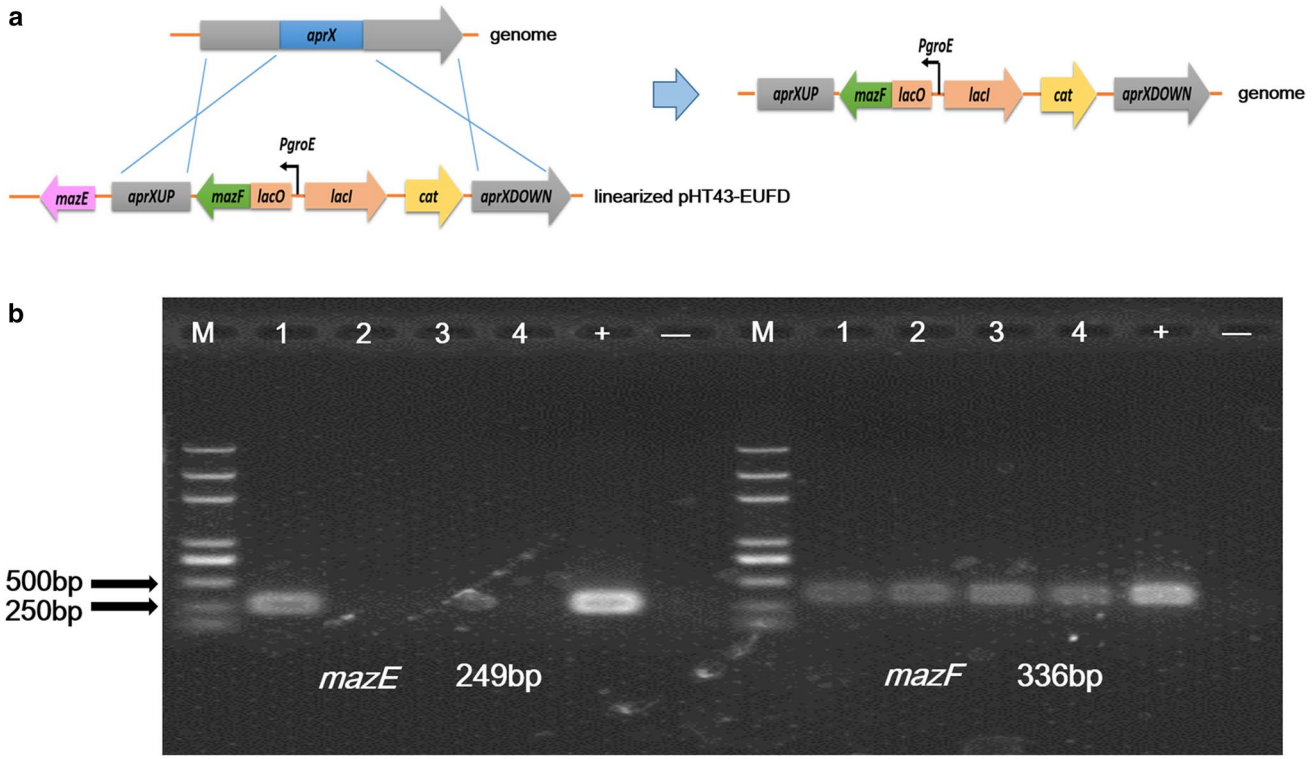

Fig. 2 Construction of *B. subtilis* F-168 (**a**) and PCR identification of the recombinant clones (**b**). **a** Linearized plasmid pHT43-EUFD was inserted into the genome of *B. subtilis* 168 through double crossover events at the *aprx* locus. **b** PCR results using P7/P8 (*mazE*) and P9/P10 (*mazF*) as primers, respectively. M: DNA marker; 1: the negative clone; 2, 3, 4: the positive clones; +: using pHT43-EUFD as the template; −: using the genome of *B. subtilis* 168 as the template

$$\text{The transformation efficiency of recipient cells} = \frac{R2}{T2},$$

*T*1: Total amount of donor cells spread on the non-selectable plates

*T*2: Total amount of recipient cells spread on the non-antibiotic plates

*R*1: Residual donor cells on the selectable plates

*R*2: Recipient transformants on the selectable plates

Results and discussion

Construction of *B. subtilis* F-168 containing counter-selectable marker *mazF*

The *mazF* gene comes from *E. coli* and encodes an endoribonuclease MazF, which is lethal to bacteria by specifically cleaving free mRNAs at ACA sequences [7, 26]. In this work, a double crossover plasmid pHT43-*mazE*-aprxUP-*mazF*-cassette-aprxDOWN (pHT43-EUFD) was constructed by the procedure described in "Materials and methods" (Fig. 2a). To prevent the host death caused by MazF, an antitoxic gene *mazE* was introduced at the upstream of the *mazF*-cassette (Fig. 2a). After double crossover, the *mazF*-cassette containing *mazF-lac operon-cat* with the full length of 3131 bp were successfully inserted into the genome of *B. subtilis* 168 (Fig. 2a). The double crossover mutants were selected and identified by specific PCR. The positive transformants were obtained through PCR results which show the *mazF* gene band and without the *mazE* gene band (Fig. 2b). The positive transformant of *B. subtilis* 168 containing the *mazF*-cassette in its genome was named *B. subtilis* F-168 and will be used as the plasmid donor in the subsequent experiments. The expression of the toxic protein MazF was induced by IPTG for counter selecting the transformants in the plasmid transformation. After the plasmids transferring from the donor strains (*B. subtilis* F-168) to the recipient strains, the donor strains were death by IPTG inducing which facilitate the screening of the positive recipient strains.

To further determine the lethal effects of the MazF in *B. subtilis* F-168, IPTG with a concentration of 1 mM was added into the culture medium at the beginning of cultivation. Different to the control (do not add IPTG), the *B. subtilis* F-168 begin to death at the end of logarithmic phase at 12–20 h due to the expression of toxic protein MazF (Fig. 3a). When *B. subtilis* F-168 was cultured at different inducing temperatures, the CFU of surviving stains showed

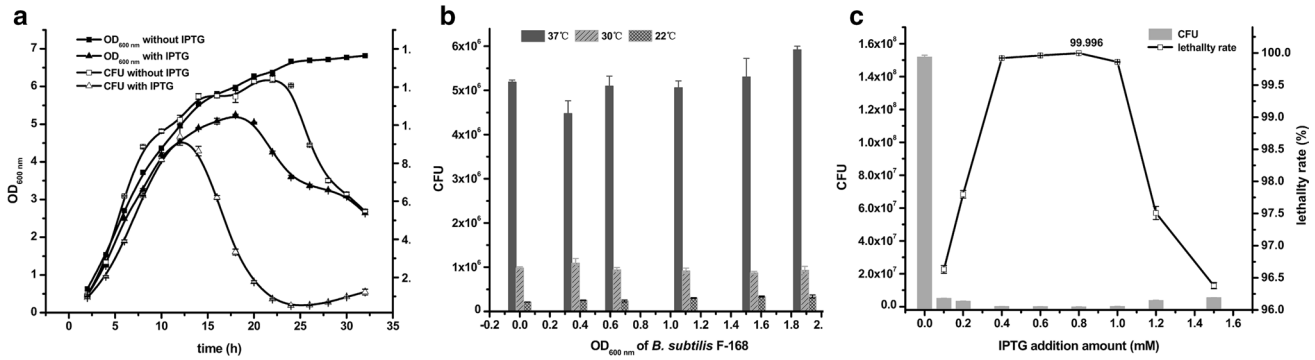

Fig. 3 Lethal effects of *B. subtilis* F-168 in different inductive conditions. **a** Growth curve of *B. subtilis* F-168 with and without IPTG. **b** Different inducing temperatures on the lethal effects of *B. subtilis* F-168. **c** Different addition amounts of IPTG on the lethal rates of *B. subtilis* F-168

obviously difference. At lower culture temperature (22 °C), the least CFU of *B. subtilis* F-168 was obtained with the highest lethality rate (Fig. 3b). Furthermore, the different lethality rates of *B. subtilis* F-168 were obtained according to various addition amounts of IPTG at 22 °C. After 24 h of cultivation, the highest lethality rate of 99.996% was reached at the IPTG concentration of 0.8 mM (Fig. 3c). When the additional amounts of IPTG were lower or higher than 0.8 mM, the lethality rate of *B. subtilis* F-168 began to decline (Fig. 3c).

These results demonstrate that the *mazF* gene from *E. coli* was a highly effective counter-selectable marker to eliminate the plasmid donor strain *B. subtilis* F-168 after plasmid interspecific transformation. So the positive recipient strains could easily be selected under the resistance of plasmid among the mix strains. The positive recipient recombinants also could be selected under double-resistance system. However, to implement double-resistance screening, it should prior import an appropriate resistant gene to the recipient strains. While most wild *Bacillus* strains do not have innate-resistant gene and hard to make molecular manipulate. Thus, the counter-selectable marker (*mazF*) makes this novel plasmid transfer method more suitable to transfer heterogeneous plasmids into the wild *Bacillus* strains which do not have any selectable markers or hard to manipulate in regular ways.

The high electrotransformation efficiency of pBE980 to *B. subtilis* F-168

B. subtilis 168 is a potential and attractive host for the transformation of heterologous plasmids. The high transformation efficiency of *B. subtilis* 168 was up to 1.0×10^4–1.0×10^6 transformants per microgramme plasmid DNA by electroporation method [9, 23]. Theoretically, the donor strain *B. subtilis* F-168, which derived from *B. subtilis* 168, could achieve the same level of transformation efficiencies of *B. subtilis* 168 using electroporation. However, it also determined by the qualified plasmid. pWB980 derived from pUB110 is commonly used for expression and secretion studies in *B. subtilis* [22]. Due to the smaller size, pWB980 could obtain relative higher electrotransformation efficiency than that of pUB110 (Table 2). While the connected products between pWB980 and the objective genes revealed much lower electrotransformation efficiency, even hard to obtain transformants. To overcome this defect, a novel shuttle plasmid pBE980 was constructed by inserting *ori* gene of pUC19 to the upstream of P43 promoter in pWB980 (Fig. 1). The electrotransformation efficiency and copy number of pBE980 to *B. subtilis* F-168 were 1.28×10^4 and 108, respectively (Table 2). Through plasmid enrichment in *E. coli* DH5α, the pBE980 and its recombinant plasmid pBE980::*pulA* and pBE980::*HSPA* could obtained the similar high electrotransformation efficiency with pWB980 to *B. subtilis* F-168 (Table 2). And the copy numbers of these plasmids are also high in the *B. subtilis* F-168 cells (Table 2) which ensure the required amount of the donor plasmids. All of these virtues provide the prerequisite for the plasmid interspecific transformation approach.

Table 2 Features of different plasmids to *B. subtilis* F-168

Plasmid	Electrotransformation efficiency[a]	Copy number
pUB110	5.16×10^3	46
pWB980	2.32×10^4	112
pBE980	1.28×10^4	108
pBE980:: *pulA*	8.6×10^3	84
pBE980::*HSPA*	1.02×10^4	92
pHT43	0.67×10^2	3

[a]The electrotransformation efficiency was determined by the numbers of the positive transformants through electroporation of one microgramme plasmid DNA

Plasmid interspecific transformation from *B. subtilis* F-168 to the wild strain *B. amyloliquefaciens* 66

B. amyloliquefaciens 66, a wild-type strain, with an attractive ability for secreting proteins was used as a plasmid recipient strain. And the plasmid pBE980 and its recombinant plasmid pBE980::*pulA* were successfully transferred from *B. subtilis* F-168 to *B. amyloliquefaciens* 66 according to the methods described in "Materials and methods", respectively. The positive recombinant recipient clones were selected by their colonial morphologies (Fig. 4a, b). All the selected positive recipient strains could extract the target plasmids (Fig. S1c). To further identified the positive recipient stains, PCR for 16s DNA and desired gene (*pulA*) were used. The results showed that all the selected positive recipient strains were *B. amyloliquefaciens* 66 (data not shown). And the positive recipient strains *B. amyloliquefaciens* 66 (pBE980::*pulA*) have *pulA* gene, but do not have the *mazF* gene (Fig. S1a, b). Moreover, the amount of positive transformants harboring pBE980 was up to 3.4×10^3 which was a little higher than that (1.3×10^3) of transformants harboring pBE980::*pulA* (Fig. 4c). It may indicate that the efficiency of this plasmid interspecific transformation is closely related with the size of plasmid. The interspecific transformation efficiencies of plasmid pBE980 and pBE980::*pulA* were 1.1×10^{-5} and 2.8×10^{-5}, respectively. In these results, plasmid pBE980 and its recombinant plasmid (pBE980::*pulA*) have proved to be feasibility of interspecific transformation from *B. subtilis* F-168 to the wild strain *B. amyloliquefaciens* 66. And those also illustrated that the interspecific transformation with counter-selectable marker *mazF* could screen the transformants without antibiotic selectable marker in recipient strains. That could provide convenient for wild *Bacillus* strains as the recipients. However, the transformation efficiency of the plasmid interspecific transformation still has room for improvement.

Optimization of plasmid interspecific transformation

Through previous study, some key factors such as the growth stage of the donor cells before co-culturing, the growth stage of the recipient cells before co-culturing, the mixed volume ratio and the co-incubation time could affect the interspecific transformation efficiency at different levels. To further improve the interspecific transformation efficiency, a series of transformation conditions were optimized.

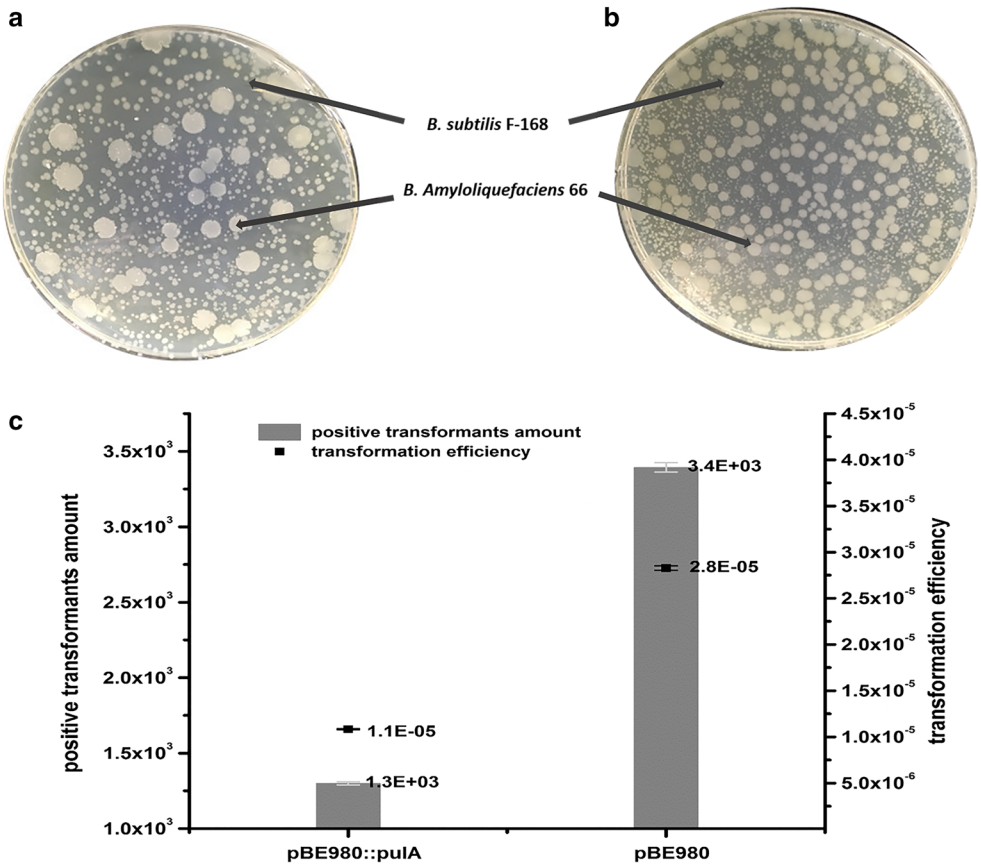

Fig. 4 Plasmid interspecific transformation from *B. subtilis* F-168 to *B. amyloliquefaciens* 66. **a** Transformants harboring pBE980:: *pulA*; **b** transformants harboring pBE980; **c** interspecific transformation efficiency of pBE980 and pBE980::*pulA*, respectively

Effects of the growth stage of the donor cells before co-culturing

The donor strain *B. subtilis* F-168 (pBE980::*pulA*) were cultured previously to different OD_{600nm} (from 0.3 to 2.0). And then were mixed with the recipient strain *B. amyloliquefaciens* 66 in its OD_{600nm} at 0.85 and the volume ratio was 5:5. After co-incubating at 22 °C and 220 rpm for 12 h, the mixed cells were properly diluted and coated on the selected plates. In the results, when the OD_{600nm} of donor cells were between 1.3 and 1.7, which was at the later of exponential stage of the cells, the positive transformants were higher than 1.5×10^4 (Fig. 5a). The highest transformant amount was up to 2.38×10^4 at $OD_{600nm} = 1.387$, while the interspecific transformation efficiency was up to 1.98×10^{-4} correspondingly (Fig. 5a). It probably because the plasmids were more facile to be released into the extracellular at the later of logarithmic phase (Fig. S2a). When the cells in the later of the logarithmic phase were inoculated into fresh medium, more plasmids were free in the solution resulting in high interspecific transformation efficiency to the recipient cells.

Effects of the growth stage of the recipient cells before co-culturing

When the donor cells were cultured to $OD_{600nm} = 1.4$, various growth stages ($OD_{600nm} = 0.3$–1.5) of the recipient cells were mixed with those at volume ratio of 5:5. The mixed strains were then co-cultured at 22 °C and 220 rpm for another 12 h. The positive transformants were at least 1.5×10^4 when the OD_{600nm} of recipient cells before co-culturing was 0.5–0.9 (Fig. 5b). And the optimal OD_{600nm} of recipient cells was 0.85 which lead to the highest positive transformants amount of 2.22×10^4 and the highest interspecific transformation efficiency of 1.85×10^{-4} (Fig. 5b). In this experiment, we conclude that the recipient cells were inoculated at the early stage of the logarithmic phase ($OD_{600nm} =$ 0.5–0.9) could

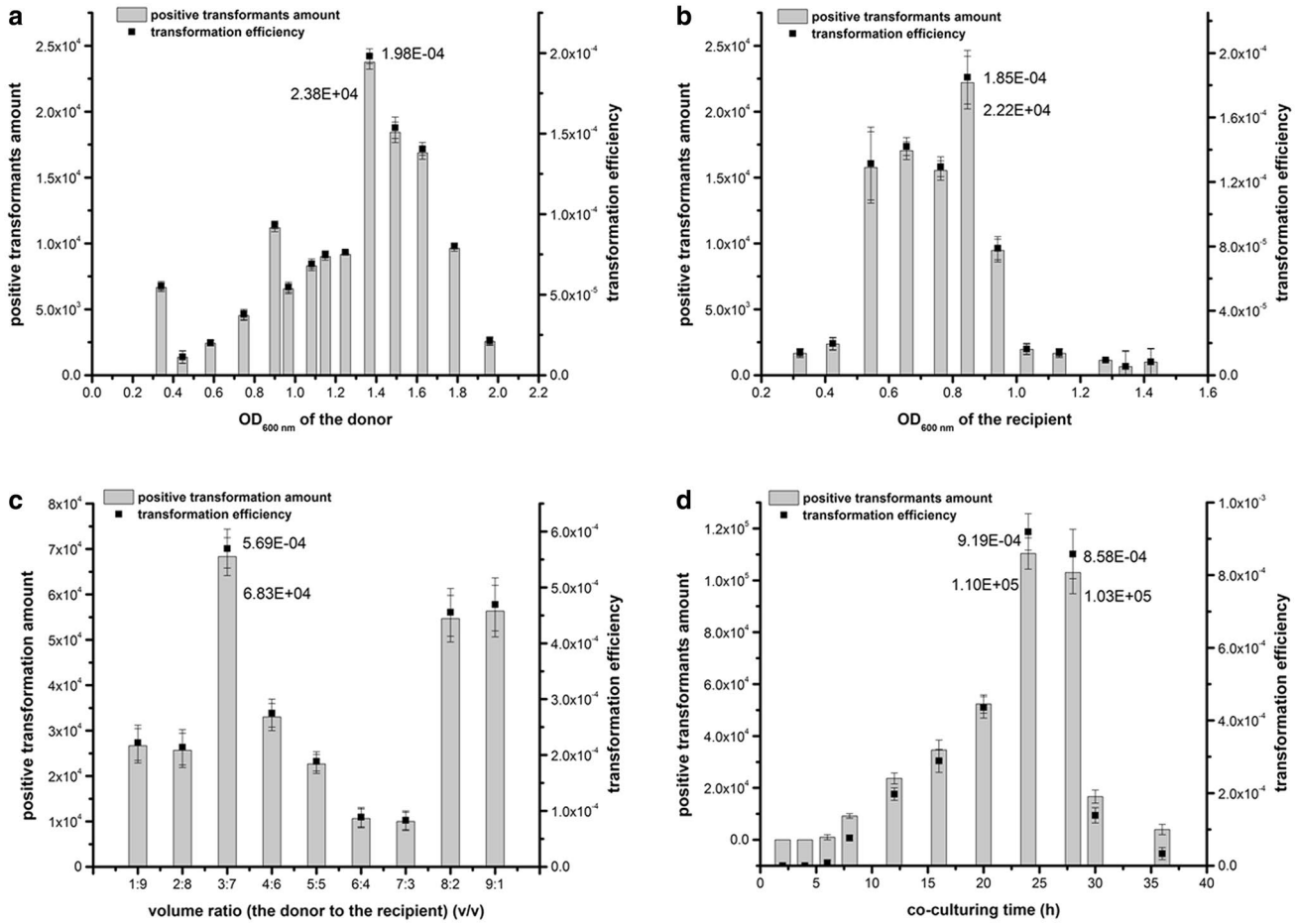

Fig. 5 Optimization of plasmid interspecific transformation from *B. subtilis* F-168 to *B. amyloliquefaciens* 66. **a** Effects on interspecific transformation efficiency by different OD_{600nm} of the donor cells. **b** Effects on interspecific transformation efficiency by different OD_{600nm} of the recipient cells. **c** Effects on interspecific transformation efficiency by different volume ratios of the donor to the recipient in the mixture. **d** Effects on interspecific transformation efficiency by different co-incubation times of the donor and recipient mixture

facilitate absorption of exogenous plasmids due to their active growth (Fig. S2b).

Effects of the volume ratio of the donor to the recipient in the mixture

Before the two kinds of cells were mixed, the donor *B. subtilis* F-168 (pBE980:: *pulA*) and the recipient *B. amyloliquefaciens* 66 were cultured to OD_{600nm} = 1.4 and OD_{600nm} = 0.85, respectively. And then they were mixed at different volume ratios (1:9; 2:8; 3:7; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1) to the total volume of 200 µl. For co-incubating, the 200 µl mixed cells were incubated into 1.8 ml fresh LB medium with 0.8 mM IPTG. The culture condition was also at 22 °C and 220 rpm for 12 h. The most positive transformants (6.83×10^4) were obtained at a volume ratio of 3:7 (the donor to the recipient, v/v). And at that ratio, the highest interspecific transformation efficiency was up to 1.85×10^{-4} (Fig. 5c). Though the volume ratio of 8:2 and 9:1 also showed relative high interspecific transformation efficiency (Fig. 5c), the residue donor cells simultaneously occupied a high proportion in the mixed cells which would hinder the screen of positive recombinant. Thus, the optimum mix ratio was not only lie on the high-positive transformants but also need to consider the convenient for positive recipient cells selection.

Effects of co-incubation time of the donor and recipient mixture

Based on the determined transformation condition above, different co-culturing times from 2 to 36 h were test for the higher interspecific transformation efficiency. The results indicated that the transformation amount reached to 1.0×10^5 after co-cultured for 24–28 h (Fig. 5d). The highest positive transformants amount and the highest interspecific transformation efficiency were 1.10×10^5 and 9.19×10^{-4}, respectively, when they co-cultured for 24 h (Fig. 5d). Moreover, the interspecific transformation efficiency was increasing gradually in the co-cultured time from 12 to 24 h. It indicates that the plasmid interspecific transformation in this study needs a relatively long period of incubation time. The process of plasmid transfer was during the growth of the mix strains.

The plasmid interspecific transformation system for various wild *Bacillus* species

Based on the optimized transformation conditions above, *B. subtilis* F-168 was further used as the donor to introduce plasmids pBE980, pBE980::*pulA* and pBE980::*HSPA* into *B. licheniformis* 124 and *B. megaterium* 258 through the interspecific transformation, respectively (Table 3). The total flowchart of the plasmid interspecific transfer method was shown in Fig. 6. After co-cultured for 24 h at 22 °C in LB medium containing 0.8 mM IPTG, the mixture was plated on selective plates (kan25 + 0.8 mM IPTG) with an appropriate dilution (Fig. 6). The positive transformants were confirmed by plasmid extraction and PCR identification using primers 980S/980A. Comparing the recombinant plasmids pBE980::*pulA* and pBE980::*HSPA*, pBE980 showed the highest interspecific transformation efficiency to the three recipient *Bacillus* species, respectively (Table 3). In a word, the plasmid interspecific transformation method was confirmed to be valid at least to the three wild-type recipient *Bacillus* strains in this study. It can be largely solved the difficulty in transferring plasmids into the three wild-type strains. Moreover, many *Bacillus* species are of considerable interest to industry and agriculture [30]. Both gene function research and strain improvement require effective plasmid transfer method for genetic manipulation in these species. Despite the fact that a number of plasmid-borne genes have been cloned and expressed in *Bacillus* species, genetic studies on wild *Bacillus* species have been seriously hampered by the lack of efficient methods for the plasmid transformation. Various studies have reported the transfer of plasmid DNAs into *Bacillus* species by biochemical transformation, electroporation, and protoplast method. While these methods suffer from serious shortcomings such as cumbersome and very low transformation efficiencies [9]. In this work, a simpler whole cell operate procedure has been achieved,

Table 3 Comparison of the interspecific transformation efficiency of different plasmids to various recipient *Bacillus* strains

Plasmids	Strains	Interspecific transformation efficiency[a]
pBE980	*B. amyloliquefaciens*	1.3×10^{-3}
	B. licheniformis	1.0×10^{-3}
	B. megaterium	5.2×10^{-4}
pBE980:: *pulA*	*B. amyloliquefaciens*	9.2×10^{-4}
	B. licheniformis	5.9×10^{-4}
	B. megaterium	6.1×10^{-5}
pBE980::*HSPA*	*B. amyloliquefaciens*	1.0×10^{-3}
	B. licheniformis	8.4×10^{-4}
	B. megaterium	8.2×10^{-5}
pWB980	*B. amyloliquefaciens*	2.9×10^{-3}
	B. licheniformis	1.0×10^{-3}
	B. megaterium	6.8×10^{-4}
pHT43	*B. amyloliquefaciens*	0
	B. licheniformis	0
	B. megaterium	0

[a]The interspecific transformation efficiency was determined by the proportion of the positive recipient transformants (on the selectable plates) against the total amount of recipient cells (on the non-antibiotic plates)

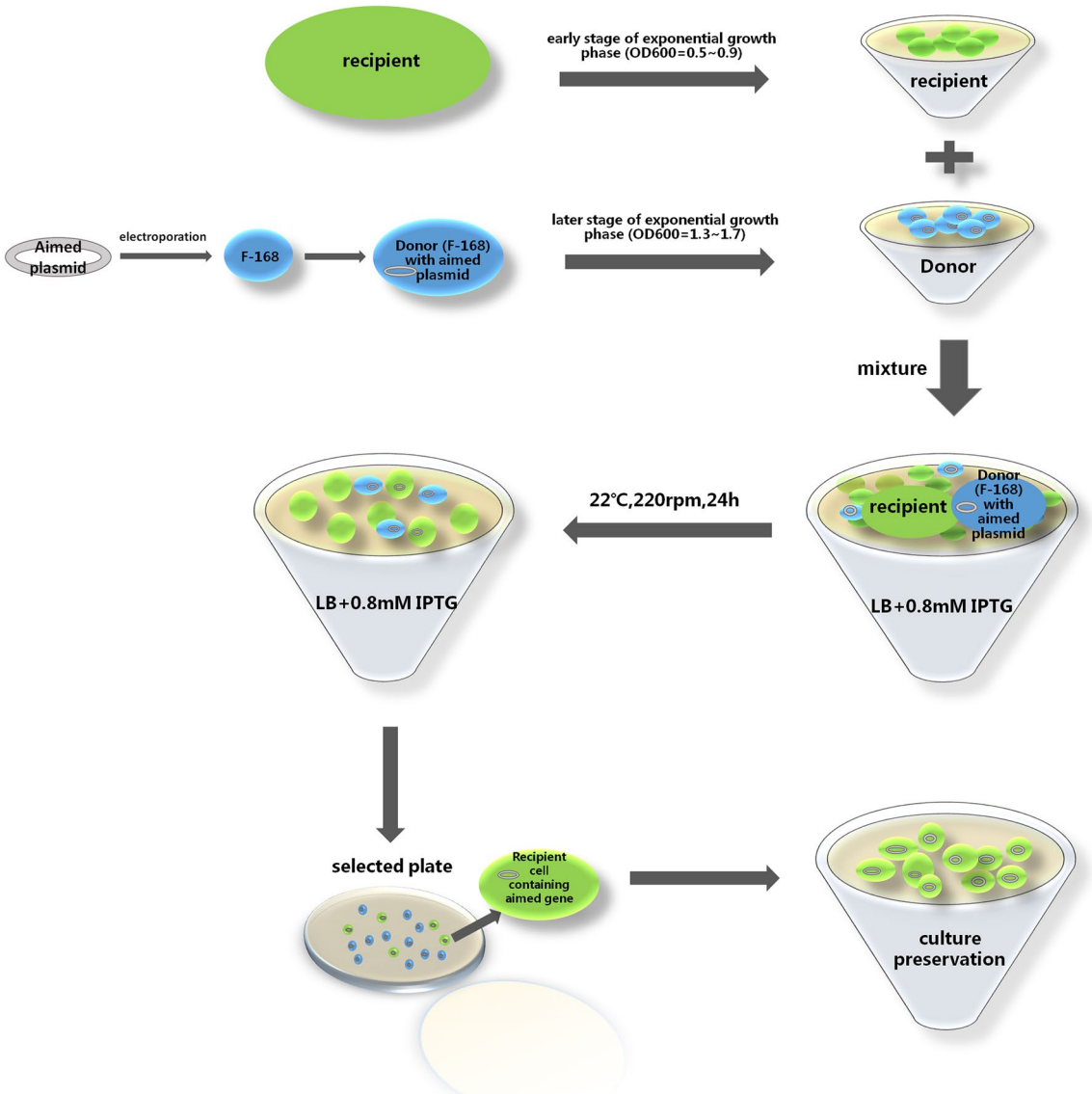

Fig. 6 The procedure of the plasmid interspecific transformation

which do not need a complicated process of making competent cells (Fig. 6). However, the detailed transfer mechanism remains to be further research.

The plasmid pBE980 was an *E. coli/B. subtilis* shuttle plasmid compounding from pWB980 and pUC19. And the *B. subtilis* plasmid pWB980 was derived from pUB110 with destroyed *mob* region [22]. Thus, the plasmid pBE980 has no autogenous transfer ability in this study. And it have no mediated plasmids been found neither in the donor strains nor in the recipient strains. The result of control experiment, which mixed the plasmid DNAs and the recipient cells under the same procedure, showed that the plasmids could not transfer directly to the recipient strains. It indicated that the novel plasmid interspecific transformation method reported here mainly based on natural transfer system of the donor and the recipient strains. The plasmids pWB980 and pHT43 were also tried to perform interspecific transformation under the optimum co-culture conditions, respectively. As a result, pWB980 showed little higher transformation efficiencies to all of the three recipient strains than that of pBE980 (Table 3). However, pHT43 could not be obtained any positive recipient transformant (Table 3). The necessary optimization of the transformation conditions has also been performed and resulted in no improvement (data not shown). But when *B. subtilis* WB600 was used as the recipient cells, a very low transformation efficiency (1×10^{-6}) of pHT43 was obtained using this method. So the main reason may be that pHT43 cannot replicate in those different species strains. Besides, the lower copy number (Table 2) of

pHT43 and the different replicate mode (θ-type replicate mode) [12, 19] with pWB980 were possibly resulted in the low transformation efficiency. Fortunately, the plasmid pBE980 used in this work showed well compatibility in all these selected strains. And these three strains have typical evolutionary relationship with the most of the type or industrially important *Bacillus* strains (Fig. S3). Furthermore, the derivative plasmids of pUB110 are still the main current *Bacillus* research tools. So the pBE980 with the replicon derived from pUB110 used in this work has certain representativeness. As to whether other plasmids (pUB110 derivative or not pUB110 derivative) could be transformed using this method, the replication ability in different *Bacillus* species strains is prerequisite and needs to be further studied. Moreover, according to a previous report, an aggregation phenotype of *Bacillus thuringiensis* subsp. israelensis has supposed to be correlated with conjugation-like plasmid transfer [6]. In this study, the aggregation phenotype and the growth periods of both donor and recipient strains also showed important effect on the natural plasmid transfer phenomenon.

Conclusion

This study constructed a novel, efficient and convenient plasmid interspecific transformation method to solve the puzzle for plasmid transformation to the wild-type *Bacillus* strains. This approach expands the alternatives of the traditional transformation methods and makes the more successful plasmid transformation into the wild-type *Bacillus* strains become possible. Compared with traditional transformation methods, this new method has three superiorities. First, it revealed high transformation success rate to the wild-type *Bacillus* strains, while the traditional transformation generally difficult to achieve. Second, the manipulation is simple and convenient. It needs no preparation of competent cells. It can be performed under normal cultivating process. Third, it showed wider applicable range in recipient *Bacillus* species such as *B. amyloliquefaciens*, *B. licheniformis* and *B. megaterium*. These features make it an attractive technique to facilitate genetic studies and industrial application of these *Bacillus* species. And this technique may prove to be applicable to other members of the genus *Bacillus*.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

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