Full Paper

Optimization of RK2-based gene introduction system for *Bacillus subtilis*

(Received November 15, 2018; Accepted November 30, 2018; J-STAGE Advance publication date: June 5, 2019)

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The Gram-positive bacterium *Bacillus subtilis* plays important roles in both industrial applications and basic research. However, transformation of competent *B. subtilis* cells is more difficult to achieve compared with that of *Escherichia coli*. It has been reported that the conjugative broad host range plasmid RK2 can be transferred to various organisms, including *B. subtilis*. Nevertheless, the protocol for conjugation from *E. coli* to *B. subtilis* has not been properly established. Thus, we optimized interspecies conjugation from *E. coli* to *B. subtilis* using the RK2 system. We constructed mobilizable shuttle and integrative vectors pEB1 and pEB2, respectively. pEB1 was used to evaluate the effect of mating media, time, temperature, and genetic background of the recipient and donor strains. We found that conjugation was not significantly affected by the conjugation time or genetic background of the recipient and donor strains. Conjugation on agar was more efficient than that in a liquid medium. A low temperature (16°C and lower) drastically decreased conjugation efficiency. When using the optimized protocol for homologous recombination after conjugation, we could not obtain double crossover mutants, as only single crossover mutants were observed in the initial selection. We then established a two-step homologous recombination method whereby positive colonies were cultivated further, which finally allowed efficient yield of double crossover recombinants. The optimized conjugation method described here allowed facility and efficient gene introduction into *B. subtilis* from *E. coli*.

Key Words: *Bacillus subtilis*; conjugation; optimization; RK2

Introduction

Bacillus subtilis is a Gram-positive bacterium widely used in basic research, industrial fermentation, and the traditional preparation of fermented food. Despite these favorable characteristics, genetic manipulation systems, that would include transformation, have been developed only for few well-characterized laboratory strains, such as *B. subtilis* Marburg 168 and PY79. The natural transformation method (Zhang et al., 2014) polyethylene glycol-mediated protoplast transformation (Tichý et al., 1968), and electroporation method (Masson et al., 1989) have been utilized to allow DNA transfer into *B. subtilis*, but they are not as easy as in *E. coli*. In addition, these three methods have DNA size limitation due to shear stress and DNA passage during transformation.

Bacterial conjugation is an intercellular DNA transfer process that requires direct contact between donor and recipient cells. This phenomenon has been implicated in sex determination of *E. coli* (Arutyunov and Frost, 2013), horizontal gene transfer, and spread of toxic or antibiotic resistance genes in nature (Cabezón et al., 2015). Broad host range conjugative plasmids, such as R factor (Resistance transfer factor), promote conjugation between various organisms. In addition, horizontal gene transfer of R factor is assumed to generate drug-resistant bacteria that cause problems in clinical establishments (Levy, 2002). RK2, a multidrug resistant broad host range R plasmid isolated from *Pseudomonas aeruginosa* S8 (Meyer et al., 1975; Saunders and Grinsted, 1972), is self-transmissible and classified as an incompatibility group P (Kolatka et al., 2010). RK2 maintained stably in some bacteria species and could mobilize the other plasmid having RK2 *oriT* simultaneously. In addition, RK2 has a capability of transferring DNA from *E. coli* to various organisms, including Gram-positive bacteria (Dominguez and O'Sullivan, 2013; Mazodier et al., 1989; Trieu-Cuot et al., 1991), yeast (Nishikawa et al., 1990), and CHO cells (Waters, 2001),

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None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Table 1. Strain and plasmid used in this study.

Strains	Description (genotype)
<i>E. coli</i> DH10B	F ⁺ , mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80dlacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara leu)7697, galU, galK, λ-, rpsL, endA1, nupG
<i>E. coli</i> BW38244	F ⁺ , RP4-2(TetS, kan1360::FRT), Δ(araD-araB)567, ΔlacZ4787(::rrmB-3), ΔdapA1341::[erm pir+], rph-1, Δ(rhaD-rhaB)568, hsdR514
<i>E. coli</i> S17-1 ΔMuRL	F ⁺ , thiE1 E1, pro-82, hsdR, recA1, endA1, RP4-2 (ΔMuRL Km::Tn7 (Tp Sm)), (LAMPir)
<i>B. subtilis</i> BEST310	<i>B. subtilis</i> RM125 derivative, containing the pBR::cl-spc and pR-neo cassettes
<i>B. subtilis</i> BEST2142	<i>B. subtilis</i> Marburg 168 derivative, recA::spc
<i>B. subtilis</i> BEST8504	<i>B. subtilis</i> Marburg 168 derivative, srf+
plasmid	description
pUB307	RK2 derivative, Tn1 deletion mutant
PHY300PLK	Shuttle cloning vector, contains p15a and pAMα1 origin
pCISP401	pBR322 derivative, integrative vector for <i>B. subtilis</i> BGM system
pEB1	PHY300PLK contains functional RK2 oriT sequence
pEB2	pCISP401 contains functional RK2 oriT sequence same as pEB1

as well as to gram-negative bacteria without a specific receptor on the recipient.

In 1991, it was reported that gene introduction into *B. subtilis* by RK2-mediated conjugation (Poyart and Trieu-Cuot, 1997; Trieu-Cuot et al., 1991). They showed DNA transfer from *E. coli* harboring an RK2-derived plasmid to *B. subtilis* BM4186 and NEM314. Notably, the efficiency of transfer was significantly different between the two recipient strains. These results suggest that the conjugation efficiency varies depending on the genetic background of the recipients. In addition, mating protocol has not been well defined in those previous studies. Thus, to apply RK2 conjugation system for transformation of various organisms, mating conditions should be established and a mating protocol has to be validated. For this purpose, in the present study, we optimized conjugation of RK2 from *E. coli* to *B. subtilis*, examined mating in liquid and agar-based media, as well as determined the effects of mating time, temperature, and donor/recipient genetic background on the conjugation efficiency.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used are listed in Table 1. *B. subtilis* and *E. coli* were grown in LB or on LB agar plates at 37°C. The antibiotics ampicillin (Ap: 100 μg/mL), tetracycline (Tet: 10 μg/mL), kanamycin (Km: 25 μg/mL), and spectinomycin (Spc: 50 μg/mL) were added to the media if needed. Diaminopimelic acid (DAP) was used for post-conjugational counter selection at a final concentration of 0.3 mM. All chemicals used in this study were purchased from Nacalai Tesque (Kyoto, Japan).

Conjugation method. Quantitative mating assay was used to estimate the efficiency of DNA transfer from *E. coli* to *B. subtilis*. Donor and recipient strains were grown at 37°C overnight in LB broth that contained appropriate antibiotics. The cells were washed twice by fresh LB and inoculated into LB medium at a final concentration of 2% and grown to mid log phase (OD₆₀₀ = 0.4–0.6). For conjugation in a liquid media, 1 ml of donor and 100 μl of recipient cells were mixed and concentrated to 100 μl, then the concentrated cell mixture was put into 2 ml LB medium. For conjugation on solid media, the same 100 μl cell mixture were spread on 3 cm × 3 cm nylon membrane put on LB agar plate and incubated at various temperatures. Mated cells were harvested from the mixed liquid culture or the nylon membrane to pre-chilled LB broth by vortexing and serial dilutions were spread on selective LB agar plates containing appropriate antibiotics. Conjugation efficiency was calculated as the average number of transconjugants per number of recipient cells.

Construction of a mobile vector. To optimize the conditions of mating between *E. coli* and *B. subtilis*, the PHY300PLK-based mobile shuttle vector pEB1 was constructed. The *NotI* fragment containing complete *oriT* was amplified from pUB307 by PCR with primers oriT_NotI_FXba: GTTCTAGAGCGGCCGC-CTGCGCGATCCGCTCC and oriT_NotI_REco: GCGGAATTTCGCGGCATGGTGTGTTTCGCGGCCGC, and the amplified fragment was cloned into PHY300PLK

Fig. 1. Comparison of conjugation efficiency under different mating conditions.

pEB1 from *E. coli* DH10B harboring pUB307 was transferred to *B. subtilis* BEST310 by conjugation on a solid or in liquid media. A. Mating on nylon membrane. B. Mating in liquid LB medium. Conjugation efficiency was calculated as the ratio of the number of transconjugants (tetracycline resistant (Tet^R) and spectinomycin resistant (Spc^R)) to the total number of recipient cells (Spc^R). No significant effect of conjugation time on conjugation was observed at either condition. Conjugation efficiency in a liquid culture was significantly lower than that on a nylon membrane. Data are presented as the mean $\pm$ standard error (SE); $n > 5$.

through *Xba*I and *Eco*RI sites after digestion of the restriction enzymes. To test the efficiency of HR mediated by transferred DNA, the pCISP401-based mobile vector pEB2 was constructed. pCISP401 contained pBR322 replication origin that could be maintained only in *E. coli*. The *oriT* fragment, same as in pEB1 was cloned into pCISP401.

Results and Discussion

Optimization of conjugation conditions

In previous studies, RK2-based bacterial conjugation was usually performed on a solid surface, using a membrane on an agar plate. In *E. coli*, RP4-mediated conjugation proceeds with approximately a four orders of magnitude higher efficiency on agar than in a liquid medium (Samuels et al., 2000). But the conditions for interspecies conjugation, including that between *E. coli* and *B. subtilis*, have not been properly established.

To evaluate the efficiency of interspecies conjugation in a liquid medium and on agar, we tested RK2-mediated conjugative transfer of pEB1 from *E. coli* DH10B harboring pUB307 Tn5 to *B. subtilis* BEST310 in LB broth and on a nylon membrane on LB agar. As shown in Fig. 1A, the conjugation efficiency on a nylon membrane was $1.2\text{--}1.8 \times 10^{-3}$ transconjugants per recipients and the maximal efficiency was observed in mating for 1 h. This maximum value was 234-fold higher than the highest conjugation efficiency in liquid LB after 5 h conjugation (7.7×10^{-6} , Fig. 1B). This result clearly indicated that cell mobility during conjugation significantly influenced transfer efficiency, and that conjugation on a membrane was a better method of RK2-mediated DNA transfer to *B. subtilis*.

During bacterial conjugation, direct contact between cells is required to make conjugative junctions for DNA transfer. This step is called mating pair formation and the selectivity of the recipient is determined by the type of plasmid. In the case of F plasmid, flexible pilus mediates

Fig. 2. Effect of mating temperature on the conjugation efficiency of pEB1.

Mating was performed at different temperatures. Conjugation efficiency was significantly decreased at a low temperature (below 16°C). Conjugation efficiency was calculated as the ratio of the number of transconjugants to the total number of recipient cells as in Fig. 1. Data are presented as the mean $\pm$ SE; $n > 5$.

the initial contact formation between *E. coli* cells (Samuels et al., 2000). Then, the cells establish direct contacts and subsequently make conjugational junctions (Dürrenberger et al., 1991). It has been suggested that cells aggregating during F conjugation after pilus binding form a stable complex, even in the liquid medium (Achtman, 1975). However, analysis of RK2 conjugation between *E. coli* cells suggested that the pilus is not essential for cell-to-cell contact, but is required for DNA transfer (Samuels et al., 2000). In addition, the study posited that RK2 pilus only contributes to the stabilization of conjugative junctions between cells and detaches easily from the cell surface.

In our experiments, conjugation between *E. coli* and *B. subtilis* on the membrane proceeded with a considerably higher efficiency than in the liquid phase, which supported the hypothesis that RK2 system requires stabilization for effective conjugation, although the possibility of the influence of cell density remains. Thus, we used solid phase conjugation in all subsequent experiments. Nonetheless,

Fig. 3. Effect of the recipient genetic background on transformation and conjugation of pEB1.

Bacillus subtilis Marburg 168 derived strains were used as recipients. BEST310 is a genome vector host that contains a part of pBR322 scaffold and *cl*-Spc cassette. BEST22142 is a *recA* deletion mutant strain. BEST8504 is a strain with low natural competence that has the activated *srf* gene. A. BEST8504 showed no competence in the natural transformation procedure. B. Genetic background of the recipient strain did not affect the conjugation efficiency in these three strains. Data are presented as the mean $\pm$ SE; $n > 5$.

although the efficiency of conjugation was low in the liquid phase, the number of transconjugants was sufficient for many research purposes (Fig. 1B). Thus, RK2 system may enhance analysis throughput if it is used in conjunction with liquid phase mating and library manipulations for gene knockout simultaneously in various strains.

Optimization of the conjugation time

To determine the minimal time for plasmid transfer by conjugation, we tested the effect of conjugation time using a 4.9-kbp mobile shuttle vector, pEB1. We stopped conjugation at 1, 2, 3, 4, 5, and 12 h after mix culture initiation by vortexing. As shown in Figs. 1A and B, conjugation efficiency was similar at different time points, clearly indicating that conjugation from *E. coli* to *B. subtilis* was completed in less than 1 h. In a previous study, RK2-mediated conjugal transfer from *E. coli* to other bacteria was performed by mating for longer, 12–18-h, periods (Poyart and Trieu-Cuot, 1997). However, mating for 30 min or less gave a small number of transconjugants (Fig. S1). We thought that this inefficient plasmid transfer was due to the expression of antibiotic resistance genes or incomplete DNA transfer. Generally, incubation for 45–60 min was required for antibiotic resistance expression when we introduced an exogenous plasmid into *B. subtilis* by natural transformation using the Tet selection. Hence, DNA introduction through conjugation required a very short time, within 30 to 60 min, and the expression time for Tet resistance would be crucial.

Evaluation of the mating temperature effect

To investigate the effect of temperature on mating, conjugation was performed at 4, 16, 25, 30, and 37°C. Donor and recipient cells were mixed and placed on a nylon membrane on LB agar at the respective temperatures for 1 h.

As shown in Fig. 2, conjugation efficiency values at 25, 30 and 37°C were similar. The lower mating temperature of 16°C attenuated transfer efficiency, whereas the tem-

Fig. 4. Effect of the donor genetic background on conjugation efficiency of pEB1.

E. coli strains with different genetic background were used as donors. DH10B harboring pUB307 was used as the standard donor strain in this study. S17-1 has an RK2 *tra* region in its chromosome. In BW38244 strain, *dapA* gene was replaced with *pir*. Data are presented as the mean $\pm$ SE; $n > 5$.

perature of 4°C was not suitable for conjugation between *E. coli* and *B. subtilis* (Fig. 2).

The importance of mating temperature was noted in previous reports concerning other conjugal plasmids. In the case of R27, Forns et al. (2005) reported that 25°C was the optimal temperature for conjugation between *E. coli* cells, whereas higher temperatures were associated with a lower conjugation efficiency. A study about F type R plasmid R1*drd*-19 reported that at temperatures lower than 37°C, conjugation efficiency was suboptimal (Singleton and Anson, 1983). Investigation for RK2-mediated conjugal transfer between *E. coli* and *Vibrio salmonicida* at a low temperature of 14°C reported that the shuttle vector was transferred with efficiency values between 10⁻⁴ and

Fig. 5. Schematic model of a homologous recombination event during the conjugation between *E. coli* and *B. subtilis*.

The experimental design was the *cI857-spc* region replacement with the DNA region containing the *cat* (Left) from transferred pEB2. If a double crossover (DCO) event occurred, the *cI857* would have disappeared and *neo* could be expressed (Right: lower). If a single cross over (SCO) event occurred, the repression of *neo* and the expression of *spc* would remain (Right: middle, upper). *spc*, spectinomycin resistant gene; *cat*, chloramphenicol resistant gene; *neo*, neomycin resistant gene.

10^{-5} (Valla et al., 1992). The minimal growth temperature for *E. coli* has been reported to be between 7.5 to 7.8°C (Shaw et al., 1971) and growth temperatures below 21°C induced systems failure in *E. coli* and resulted in very slow growth (Strocchi et al., 2006). In addition, protein synthesis is rapidly decreased at low temperatures due to the accumulation of 70S ribosomal subunit (Perrot et al., 2000). Given these previous findings, our result at 4°C could not exclude the possibility of conjugation during incubation on a selective plate. In addition, conjugative transfer occurred at 16°C, and a comparatively large amount of DNA was introduced into *B. subtilis*. The fact that conjugative transfer from *E. coli* to *B. subtilis* occurred at 16°C was in line with the observed conjugation between *E. coli* and *V. salmonicida*.

Evaluation of the effect of the strain genetic background

Previous studies have suggested that the genetic back-

ground of recipient *B. subtilis* influences conjugation efficiency (Poyart and Trieu-Cuot, 1997; Trieu-Cuot et al., 1987, 1991). In their studies, they used a pAM β 1-derived vector that contained transfer origin of RK2 and the *B. subtilis* Marburg 168 derivative strains BM4186 and NEM314 as recipients. BM4186 is a spontaneous streptomycin-resistant mutant of MT127. MT127 has a *leuB6* mutation in addition to other features of a typical strain 168 (*trpC2*, *BsuM* *res*⁺/*mod*⁺) (Tanaka, 1979; Ward and Zahler, 1973). NEM314 is a different spontaneous streptomycin-resistant mutant of strain 168. There was a clear difference in conjugation efficiency between BM4186 (6×10^{-5}) and NEM314 (3×10^{-7}) (Poyart and Trieu-Cuot, 1997; Trieu-Cuot et al., 1991). These results postulated the possibility that a slight difference in genetic background causes a drastic alteration of conjugation efficiency.

To evaluate the effect of recipient genetic background, three *B. subtilis* strains BEST310, BEST22142, and

Fig. 6. Evaluation of homologous recombination frequency.

Homologous recombination (HR) mediated by the transferred plasmid was tested by colony counting as shown in Fig. 5. A. Single-step DCO. No colonies were found after 1–12 h. B. Single-step SCO. No double crossover transconjugants were observed after single-step HR. The single crossover experiment produced a sufficient number of recombinants, but the recombination frequency was significantly different from that achieved during conjugation of maintainable shuttle vector (grey bars of both panel). Data are presented as the mean $\pm$ SE; $n > 5$.

Fig. 7. Schematic diagram of the two-step homologous recombination method.

SCO recombinant obtained in the experiment of Fig. 6 was grown in LB liquid medium containing 5 μ g/mL chloramphenicol (Cm) overnight. Subsequently, the overnight culture was serially diluted and spread on LB agar plates containing 5 μ g/mL chloramphenicol and 5 μ g/mL neomycin (Nm). The conversion rate from SCO to DCO recombinant was calculated as follows: (Cm^R + Nm^R colonies)/Cm^R colonies. Azactam (Aza) was used for negative selection of the donor *E. coli* at a final concentration of 1 μ g/mL.

BEST8504 were used. BEST310 was used as a genome vector system; it contains a pBR scaffold sequence and a *cI-spc* cassette (*cI* repressor expression region with spectinomycin resistance) on *proB* locus (Kaneko et al., 2005). BEST22142 and BEST8504 were derivatives from *B. subtilis* 168 strain. The *recA* locus was replaced by a spectinomycin resistance cassette in BEST22142 and the *sfr* gene, which is inactivated in *B. subtilis* 168 strain, was activated in BEST8504 and, thus, the strain produced Surfactin. As shown in Fig. 3A, the natural transformation method using pEB1 gave no colonies when BEST8504 was used as recipient, whereas thousands of colonies were obtained where BEST310 and BEST22142 were used as recipient. Surprisingly, no such significant differences in conjugation efficiency were observed between BEST310, BEST22142, and BEST8504 strains (1.80×10^{-3} , 1.85×10^{-3} , 1.13×10^{-3}) (Fig. 3B). In comparison with the results of previous studies (Poyart and Trieu-Cuot, 1997;

Trieu-Cuot et al., 1991) the conjugation efficiency of all recipient strains in our case was about four orders of magnitude higher than that of NEM314. Conjugation event requires direct cell-to-cell contact between donor and recipient cells and penetration of the pilus through the cytoplasmic membrane of the recipient (Ilangoan et al., 2015). Additionally, NEM314 and BM4186 are spontaneous streptomycin-resistant mutants and therefore may contain unidentified mutations in the chromosome. The low transformation efficiency of Surfactin producing BEST8504 by expression of the *sfr* gene requires further investigation, and it would be important to understand the components of the DNA uptake machinery of the cell surface. Next, we evaluated the effect of the genetic background in donor strain by using S17-1 Δ MuRL and BW38244 strains. Donor strain S17-1 Δ MuRL was generated by elimination of Mu sequences from the *recA* deficient S17-1 chromosome by lambda Red seamless recombination system (H.

Mori, unpublished result). This chromosomal modification gave genetic stability to S17-1. BW38244 is a diaminopimelic acid (DAP) auxotroph strain in which the *dapA* gene was replaced with a *pir* gene for maintaining a plasmid carrying the oriR γ origin. This strain is useful for transconjugants selection by the DAP selection. DNA handling is relatively easy and broad application would be possible in both strains, and there was no significant difference in conjugation efficiency between them (Fig. 4). The *tra* genes were supplied as a single copy locus and small number loci in S17-1 Δ MuRL and BW38244/DH10B (pUB307), respectively. These results indicated that a single copy of RK2 *tra* gene locus was enough for conjugation, and two convenient strains could be optionally used for various experiments.

Homologous recombination by a transferred plasmid

To determine the frequency of homologous recombination (HR) induced by the transferred plasmid, integrative mobile plasmid pEB2, a derivative of integrative plasmid pCISP401, was introduced into *B. subtilis* by conjugation, and the chromosomal integration activity was evaluated. It has been reported that HR is induced with high frequency and high precision when plasmids undergo a natural uptake by *B. subtilis*. In addition, this advantageous property was used to accumulate foreign DNA in *B. subtilis* chromosome with Bacillus Genome vector (BGM) system (Itaya et al., 2000; Ogawa et al., 2015). However, characteristics of HR mediated by the plasmid transferred by the RK2 system have not been reported in *B. subtilis*.

First, we evaluated the frequency of HR by natural transformation. pCISP401 and pEB2 showed similar HR frequencies (pCISP401 plasmid, 7.2×10^2 CFU/100 ng; pEB2, 1.8×10^3 CFU/100 ng, respectively). Then, to determine the frequency of a double crossover event (DCO) homologous recombination induced by the transferred plasmid, we measured the number of recombinants that showed chloramphenicol (Cm) and neomycin (Nm) resistance (Cm^R and Nm^R, respectively). In BEST310, the expression pR-driven neomycin resistant gene (neo) is suppressed by the lambda repressor cI857 encoded the same locus as a spectinomycin resistant gene (spc). If the locus containing cI857 and spc was replaced with a chloramphenicol resistant gene (cat), the strain showed Cm^R and Nm^R because of cI857 exclusion (Fig. 5). The HR frequency was calculated as the colony number on a plate containing Cm and Nm/the colony numbers on a plate containing azactam (Aza) to exclude *E. coli* colonies.

During the initial screening, no Cm^R + Nm^R colonies were detected at any conjugation time (Fig. 6A). To evaluate the frequency of single crossover HR, we checked the colonies that exhibited Cm resistance that obtained the cat gene by a single crossover event (SCO), DCO, and nonhomologous recombination (Fig. 5). Frequency values were $6.1\text{--}9.2 \times 10^{-7}$ at various mating times, although the efficiency was significantly decreased compared with that during maintainable plasmid pEB1 transfer (Fig. 6B).

To obtain a DCO clone, we designed a two-step homologous recombination using second cultivation (Fig. 7). A candidate colony from LB agar plate containing Cm was cultivated in LB medium containing Cm overnight at 37°C.

Fully grown cultures were serially diluted and spread on a selective LB agar plate containing Cm and Nm. The rate of conversion of SCO to DCO recombinant was calculated by the following formula: (Cm^R + Nm^R colonies)/(Cm^R colonies). SCO recombinants were converted to DCO candidates at a frequency of 2.5×10^{-3} , and 89.1% of DCO candidates lost spectinomycin resistance. DCO candidate genome structures were checked by PCR. In contrast, no recombinants were observed in the *recA* deletion strain BEST22142. Recently, a *recA* inducible *B. subtilis* has been developed and used to control chromosomal integration (Ogawa et al., 2015). The recombination rate was clearly related to the expression level of *recA*: insufficient expression of the latter was associated with a low recombination efficiency. Generally, in bacteria, RecA is induced by the SOS-response when DNA damage occurs (Shimoni et al., 2009). *B. subtilis* has an additional pathway regulating *recA* expression. The function of LexA, a repressor of *recA*, is inhibited by ComK when natural competence is induced, and this results in higher intracellular *recA* levels (Haijema et al., 1996). However, in BEST310, intracellular RecA in the overnight culture was undetectable by western blotting (Ogawa, personal communication). It is expected that intracellular RecA level during conjugation is insufficient in *B. subtilis*, resulting in low HR efficiency. Our two-step method was effective in the BGM system. However, to obtain a DCO recombinant through conjugative transfer in any chromosomal locus, counter selection systems, such as a cI-pR system, would be required for easy handling.

Acknowledgments

We would like to thank Mitsuru Sato for technical assistance and advice. This work was partially supported by JSPS KAKENHI Grant Numbers JP16H02485 and JP25250028 to HM, JP25292047 to MK, and MEXT KAKENHI Grant Number JP15K14693 to MK.

Supplementary Materials

Supplementary figure is available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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