RESEARCH ARTICLE

Introduction of temperature-sensitive helper and donor plasmids into Bac-to-Bac baculovirus expression systems

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In the baculovirus shuttle vector (bacmid) system, a helper plasmid and a donor plasmid are employed to insert heterologous genes into a cloned baculovirus genome via Tn7 transposition in Escherichia coli. The helper and donor plasmids are usually cotransfected with constructed bacmids into insect cells, which will lead to integration of these plasmids into the viral genome, and hence to the production of defective virions. In this study, to facilitate the preparation of plasmid-free recombinant bacmids, we modified a set of helper and donor plasmids by replacing their replication origins with that of a temperature-sensitive (ts) plasmid, pSIM6. Using the resulting ts helper plasmid pMON7124ts and the ts donor plasmid pFB1ts-PH-GFP, a recombinant bacmid, bAcWT-PG(-), was constructed, and the transposition efficiency was found to be 33.1%. The plasmids were then removed by culturing at 37 °C. For bAcWT-PG(-), the infectious progeny virus titer and the protein expression level under the control of the polyhedrin promoter were similar to those of a bacmid constructed with unmodified helper and donor plasmids. These ts plasmids will be useful for obtaining plasmid-free bacmids for both heterologous protein production and fundamental studies of baculovirus biology.

KEYWORDS    baculovirus; Bac-to-Bac baculovirus expression system; Tn7 transposition; temperature-sensitive plasmid

INTRODUCTION

The baculovirus shuttle vector (bacmid) system, also known as the Bac-to-Bac baculovirus expression system (Luckow et al., 1993), is a powerful and widely used protein expression technology, in which eukaryotic proteins can be produced with high yields and often processed with authentic post-translational modifications (Luckow et al., 1993; Kost et al., 2005; van Oers et al., 2015). In this system, the approximately 130-kb Autographa californica nucleopolyhedrovirus (AcMNPV) genome has been integrated with a mini-F replicon and a mini-attTn7 site, so that it can replicate and be manipulated with Tn7 transposition in Escherichia coli (Luckow et al., 1993). With the assistance of Tn7 transposition proteins (TnsA–D) provided in trans by a helper plasmid pMON7124, heterologous genes that are cloned into the Tn7 transposon of a donor plasmid can be site-specifically transposed into the mini-attTn7 site of the AcMNPV bacmid bMON14272. Recombinant baculoviruses can then be generated by transfecting the constructed bacmids into insect cells, and the desired proteins can be expressed under the control of the polyhedrin promoter, one of the strongest baculovirus promoters currently known.

After the recombinant bacmids are constructed, the helper and donor plasmids remain in the host bacteria. Thus, these plasmids are always transfected along with the bacmids into insect cells during the generation of recombinant baculoviruses. Notably, it has been reported that when cotransfected with baculovirus genome into insect cells, plasmids are able to replicate, and up to 25%
of the replicated plasmids can be integrated into the viral genome and packaged into progeny virions (Wu et al., 1999). The integration of plasmids occurs via nonhomologous recombination, and can generate large deletions of the viral genome, leading to the production of defective virions.

In this study, we aimed to improve the bacmid system by modifying the helper plasmid pMON7124 and the donor plasmid pFB1-PH-GFP to be temperature-sensitive (ts), so that they could be removed after recombinant bacmid construction, by culturing at a temperature higher than 37 °C. The production of infectious progeny virus and the expression of polyhedrin from the plasmid-free bacmid were then compared with those from a plasmid-containing bacmid.

**MATERIALS AND METHODS**

**Plasmids, bacterial strains, and growth conditions**

The helper plasmid pMON7124 encodes the Tn7 transposition proteins (TnsA-D) and a tetracycline resistance gene (Barry, 1988). The donor plasmid pFB1-PH-GFP is ampicillin- and gentamicin-resistant, and the gentamicin resistance gene, along with a polh gene, an enhanced green fluorescence protein (egfp) gene, and a multiple cloning site, are located between the left and right arms of the Tn7 transposon (Wu et al., 2006). Both pMON7124 and pFB1-PH-GFP are derivatives of pBR322, so their replication origins (oris) are the same. The ts plasmid pSIM6 (Chan et al., 2007) was used as the template for amplifying the ts ori by PCR. The bacmid bMON14272 contains a kanamycin resistance gene, a lacZ gene (containing a mini-attTn7 site), and a mini-F replicon in the polh locus (Luckow et al., 1993).

*E. coli* DH5α was used to propagate and manipulate the plasmids, including pMON7124, pFB1-PH-GFP, and pSIM6, while *E. coli* DH10B was used to propagate and manipulate bacmids, including bMON14272. Bacteria containing the ts plasmids were cultured at 32 °C. Luria-Bertani (LB) medium or LB agar was used for culturing bacteria, and antibiotics were supplemented at the appropriate final concentrations: gentamicin (BD Biosciences, San Jose, United States) 10 μg/mL, ampicillin 100 μg/mL, kanamycin 50 μg/mL, and tetracycline 12.5 μg/mL (all Sigma-Aldrich, St. Louis, United States).

**Simple cloning**

Simple cloning was performed as described in a previous study (You et al., 2012), with minor modifications. First, a 2326-bp region in pSIM6, which contained a ts ori, was amplified by PCR. The primers had sequences flanking pBR322 ori added to their 5’ ends (TsOri-F: 5’-CCCTTAACGTAGTTTTCGGTCCACTGACGT-CAGACTTTTCTACCTCGTACTACAAACGTGTT-3’, sequence upstream of pBR322 ori underlined; TsOri-R: 5’-CGTTATCCACAGATTCCGGGATAACGCAG-GAAAGAACTAAGAGACAGCTGACGGGTT-3’, sequence downstream of pBR322 ori underlined). The amplified ts ori fragment was used as mega-primers for a secondary PCR method called prolonged overlap extension PCR (POE-PCR), with pMON7124 or pFB1-PH-GFP used as template. During POE-PCR, large DNA multimers were generated, which were composed of numerous ts oris and target plasmids in tandem. For the simple cloning protocol a linearized plasmid (obtained by PCR or enzyme digestion) was used as the template for POE-PCR, but we found that circular plasmids could also be used in this process. The molar ratio of template (about 100 ng) to mega-primers was 1:20, and Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, United States) was used to ensure fidelity. The POE-PCR parameters were as follows: 98 °C for 5 min, followed by 35 cycles of 10 s at 98 °C, 10 s at 60 °C, and 2 kb/min (approximately 7 min for pMON7124 and 4.5 min for pFB1-PH-GFP) at 72 °C, with a final step at 72 °C for 10 min. Then 2 μL (20 U) of DpnI restriction enzyme (New England Biolabs, Ipswich, United States) was added to the PCR product and incubated for 3 h to digest the parental plasmids. The resulting mixture was transformed into the chemically competent DH5α cells, generating pMON7124* and pFB1*-PH-GFP. The temperature sensitivity of pMON7124* and pFB1*-PH-GFP was verified by culturing with the corresponding antibiotics at 37 °C. The sequence of the Tn7 transposon in pFB1*-PH-GFP was confirmed by Sanger sequencing to ensure that no mutation would be introduced into the recombinant bacmids.

**Tn7 transposition**

Heat shock transformation of chemically competent bacteria was performed as described previously (Green and Sambrook, 2012), with an exception that the bacteria with ts plasmids were recovered and cultured at 32 °C instead of 37 °C. Approximately 200 ng of pMON7124* and pFB1*-PH-GFP were sequentially transformed into the DH10B strain harboring bMON14272. A single transformant was picked and cultured at 32 °C overnight in LB medium supplemented with kanamycin and tetracycline, then the bacteria were inoculated into LB medium and cultured overnight in the presence of kanamycin at 37 °C to remove the helper and donor plasmids. The resulting bacteria were streaked onto LB agar supplemented with 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 24 μg/mL isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin (50 μg/mL), and gentamicin (10 μg/mL), and cultured at 37 °C overnight. This allowed blue/white screening, as the
white colonies were the clones that had recombinant bacmids only, thus these were picked, and PCR was performed with primers (5'-GTTGTAAACGACGCAGTCGTAATTGTGAAATACGACTC-3' and 5'-GAGCGGATACAAATTTACACAGGAACAGCTATGAC-3') to confirm the correct insertion of Tn7. Loss of the helper and donor plasmids was confirmed by culturing with corresponding antibiotics, followed by agarose gel analysis, and PCR with specific primers (5'-GTTCTTTCTCGCGTTATCCCCT-3' and 5'-CGGTGTGAAATACCGCACAGTGCGTAAG-3'), which were designed according to the pBR322 backbone, so they could detect both helper and donor plasmids. The plasmid-containing recombinant bacmid was constructed with pMON7124 and pFB1-PH-GFP as previously described (Wu et al., 2006).

Transfection
Sf9 cells were cultured at 27 °C in TNM-FH medium supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 30 μg/mL streptomycin. Bacmid DNA (diluted in 50 μL sterile water) was prepared from 6 mL of bacterial overnight culture. Transfection was performed as described in our previous study (Wu et al., 2006). Approximately 1 × 10^6 Sf9 cells were seeded into a 35-mm dish and cultured for 2 h. Bacmid DNA was mixed with 5 μL Cellfectin II (Invitrogen, Waltham, United States), and added to the cells, which were cultured for a further 5 h, then the transfection mixture was replaced with 1 ml fresh medium. Virus titer was determined using a 50% tissue culture infective dose (TCID_{50}) end-point dilution assay in Sf9 cells.

Western blotting
Transfected Sf9 cells were collected at 72 h post-transfection (h p.t.) and subjected to SDS-15% PAGE. To detect polyhedrin expression, a rabbit polyclonal anti-polyhedrin antiserum (1:4,000; previously prepared in our lab) (Xiao et al., 2009) was used as primary antibody and a donkey anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:10,000; Amersham Biosciences) was used as secondary antibody. To detect actin expression, a mouse monoclonal anti-actin antibody (1:2,000) and a goat anti-mouse HRP-conjugated antibody (1:5,000) (both Abmart) were used as primary and secondary antibody, respectively. The levels of polyhedrin and actin were analyzed by scanning the densitometry of the bands with Quantity One software (Bio-Rad).

RESULTS

Construction of the ts helper plasmid pMON7124^ts and ts donor plasmid pFB1^ts-PH-GFP
To construct a ts helper plasmid for the bacmid system, we modified the helper plasmid pMON7124 by replacing its ori with that of a ts plasmid pSIM6. The ts ori was cloned into pMON7124 by using a PCR-based cloning method called simple cloning (You et al., 2012). Briefly, a ts ori fragment was amplified by PCR with primers that had sequences flanking the pBR322 ori at their 5' ends. As the backbone of pMON7124 was pBR322, the amplified ts ori fragment could be used as mega-primers to amplify pMON7124 by PCR. DNA multimers were formed during POE-PCR, and these were then transformed into E. coli DH5α. In E. coli, the DNA multimers were excised into unit lengths and circularized to form the desired chimeric plasmids, and the target plasmid was then generated. The resulting ts helper plasmid was named pMON7124^ts (Figure 1A). In pMON7124^ts, the insA-D genes and the tetracycline resistance gene remained intact, while the original 674-bp region containing the pBR322 ori was replaced with a 2326-bp fragment encoding a repA gene along with a ts ori. Compared with pMON7124, pMON7124^ts is a low-copy plasmid and will be lost when cultured at a temperature degree higher than 37 °C.

The donor plasmid pFB1-PH-GFP (Wu et al., 2006) was also modified to be ts. pFB1-PH-GFP encodes an egfp gene and a polh gene, so it can be used for observation of the virus replication in vitro and the detection of the protein expressed by the polh promoter. Because the backbone of pFB1-PH-GFP is also pBR322, pFB1-PH-GFP was modified using the same strategy as pMON7124 described above, resulting in a ts donor plasmid pFB1^ts-PH-GFP (Figure 1B). Expression of a heterologous protein in pFB1^ts-PH-GFP can be achieved by simply replacing the polh gene (under the control of a polh promoter) with the target gene. Moreover, it is noteworthy that simple cloning is a useful and rapid (only 2–3 days to obtain desired plasmids) method for modifying donor plasmids in the bacmid system, such as the widely used pFastBac1, pFastBac Dual, and their derivatives.

Construction of a recombinant bacmid with the ts helper and donor plasmids
Using the ts helper and donor plasmids, it took only 3 days to obtain recombinant bacmids that were not contaminated with plasmids. An example of the bacmid construction procedure is shown in Figure 1C; in this, a polh gene and an egfp gene were inserted into the AcMNPV bacmid by using pMON7124^ts and pFB1^ts-PH-GFP. First, pMON7124^ts and pFB1^ts-PH-GFP were sequentially transformed into a DH10B strain containing bMON14272. In this step, even if the bacteria were plated on LB agar supplemented with X-Gal and IPTG, it was rare to obtain white colonies. This may have been due to the low copy numbers of both pMON7124^ts and pFB1^ts-PH-GFP, but it did not affect the production of
recombinant bacmids in the final step. One transformant was picked and cultured overnight sequentially at 32 °C then 37 °C. During the culture at 32 °C, transposition occurred, and the helper and donor plasmids were then lost at 37 °C. The resulting bacteria were streaked and subjected to blue/white screening; the white colonies were the desired clones that had recombinant bacmids only. The resulting bacmid was named bAcWT-PG(-). This bacmid had no ampicillin or tetracycline resistance, as the antibiotic resistance was carried by the helper and donor plasmids. The bAcWT-PG(-) bacmid was also extracted and subjected to agarose gel analysis (Figure 2A) and PCR verification (Figure 2B). The results indicated that the helper and donor plasmids had been removed successfully. Moreover, the percentage of the white colonies was 33.1% ± 3.7%, which is about six-fold higher than that of the original bacmid system (5.2% ± 0.7%). As the donor plasmid had been removed, the production of blue colonies under antibiotic selection indicated that the Tn7 transposon was inserted into a site in the bacterial chromosome other than the mini-attTn7 site on the bacmid (Leusch et al., 1995; Sun et al., 2009). This was probably due to the existence of a functional chromosomal attTn7 site in DH10B, which would com-

Figure 1. Physical map of pMON7124ts (A) and pFB1ts-PH-GFP (B). Dashed line boxes indicate the modified regions of both plasmids. (C) The process of recombinant bacmid construction using both plasmids. First, the ts helper and donor plasmids are transformed into the DH10B strain that harbors the AcMNPV bacmid. Tn7 Transposition then occurs during culture with kanamycin (Kan') and tetracycline (Tet') at 32 °C, and the recombinant bacmid (re-bacmid) is generated. Helper and donor plasmids will be lost when the bacteria are cultured at 37 °C with only kanamycin and gentamicin (Gen') supplemented. The re-bacmid and the removal of plasmids can be verified by PCR and culture with corresponding antibiotics, respectively.

Figure 2. Confirmation of the removal of the helper and donor plasmids. (A) The bacmid DNA of bAcWT-PG(-) and bAcWT-PG(+) was extracted and subjected to 0.8% agarose gel electrophoresis. (B) PCR was performed on the bacmid DNA of bAcWT-PG(-) and bAcWT-PG(+) with a specific primer pair for both the helper and donor plasmids. pMON7124, pMON7124ts, pFB1-PH-GFP, and pFB1ts-PH-GFP were used as positive controls.
pete with the mini-attTn7 site on the bacmid for the Tn7 transposon (Leusch et al., 1995; Sun et al., 2009). In some host strains, such as DH10B::TnJ4327 (Leusch et al., 1995), the chromosomal attTn7 site has been blocked. Therefore, if such host strains are used in place of E. coli DH10B, Tn7 transposition would occur only on the bacmid, further improving the rate of white colonies (Leusch et al., 1995; Sun et al., 2009).

**Comparison of plasmid-free and plasmid-containing bacmids**

To determine whether the infectious recombinant baculovirus titer and the protein expression level were improved by using the plasmid-free bacmid, both plasmid-free and plasmid-containing bacmids were compared. The plasmid-free bacmid bAcWT-PG(-) and a plasmid-containing bacmid bAcWT-PG(+), constructed with the non-ts plasmids pMON7124 and pFB1-PH-GFP, were used to transfect Sf9 cells. Briefly, bacmid DNA was extracted from 6 mL overnight culture of each bacterial strain. Approximately 1 × 10^6 Sf9 cells were seeded into a 35-mm dish and transfected with bAcWT-PG(-) or bAcWT-PG(+). At 96 h p.t., progeny viruses were collected and the titer determined. There was no significant difference (p > 0.05) between the progeny virus titers from bAcWT-PG(-) and bAcWT-PG(+) (Figure 3A).

Cells were also collected at 72 h p.t. and analyzed by western blotting. As shown in Figure 3B, the level of polyhedrin detected in the cells transfected with bAcWT-PG(-) was similar to that in the cells with bAcWT-PG(+). These results suggest that although some of the cotransfected plasmids were able to integrate into the baculovirus genome (Wu et al., 1999), the total number of the resulting defective virions might be relatively limited, so that it was hard to detect the difference in performance between plasmid-free and plasmid-containing bacmids.

**DISCUSSION**

For direct selection of recombinant bacmids after Tn7 transposition, several conditional replication donor plasmids have been constructed, such as pMON18137 (Leusch et al., 1995) and pRCDMA (Sun et al., 2009). A few conditional replication helper plasmids have also been reported. A ts helper plasmid pSTNSK has been constructed for Tn7 transposition, but both pSTNSK and bMON14272 are kanamycin-resistant (Crepin et al., 2012), which does not allow antibiotic selection for transformants with both pSTNSK and bMON14272. Another ts plasmid, pGRG25, a hybrid of the helper and donor plasmids, has also been constructed (McKenzie and Craig, 2006), but this plasmid has a limited choice of restriction enzymes.

In the current study, we found that simple cloning is a rapid and suitable method to modify helper and donor plasmids in the bacmid system, and we constructed a ts helper plasmid pMON7124<sup>ts</sup>, which had a different antibiotic resistance from the AcMNPV bacmid, and a ts donor plasmid pFB1<sup>ts</sup>-PH-GFP, which has multiple choices of restriction sites. These ts helper and donor plasmids can also be used for other bacmids, such as the bacmids of *Bombyx mori* nucleopolyhedrovirus (Deng et al., 2000), *Helicoverpa armigera* nucleopolyhedrovirus (Wang et al., 2003; Si et al., 2007), and *Spodoptera exigua* multiple nucleopolyhedrovirus (Yang and Pang, 2007).
Our results did not show observable differences in infectious progeny virus titer and protein production between the plasmid-free bacmid and the plasmid-containing bacmid. This may be due to the different replication efficiencies between the baculovirus genome and plasmids in insect cells. The baculovirus genome replicates more efficiently than plasmids in insect cells, and only 10%–25% of replicated plasmids incorporate into the viral genomes (Wu et al., 1999). Therefore, the defective viral genomes resulting from plasmid incorporation may be only a relatively small portion of the total viral genomes, and it is difficult to detect the difference in infectious progeny virus titer and protein production with or without plasmid contamination.

In addition to its use for expression of heterologous proteins, the bacmid system has also been widely used for viral variant construction in fundamental research of baculovirus biology. In order to ensure that equal amount of bacmids are used in parallel experiments, recombinant bacmids are often required to be separated from helper and donor plasmids and precisely quantified. If plasmid DNA is cotransfected with bacmids into insect cells, the genetic uniformity of the recombinant baculovirus cannot be guaranteed. In the existing bacmid systems, the bacmid purification procedure is time-consuming and laborious, and is generally accomplished by antibiotic selection after electrotransforming *E. coli* DH10B with plasmid-containing bacmids (Wu et al., 2006). By contrast, with our ts helper and donor plasmids, it takes only 3 days from Tn7 transposition to obtain plasmid-free bacmids. Therefore, these ts plasmids will be useful for research into baculovirus biology by facilitating preparation of purified bacmids.

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**COMPLIANCE WITH ETHICS GUIDELINES**

This article does not contain any studies with human or animal subjects performed by any of the authors, and there is no competing interest.

**AUTHOR CONTRIBUTIONS**

Z.H., A.L., M.Y., W.W., and K.Y. conceived and designed the experiments; Z.H. and M.P. performed the experiments; Z.H., A.L., M.Y., W.W., and K.Y. analyzed the data; Z.H. and M.P. provided the reagents and materials essential for the research; Z.H. and K.Y. wrote the paper.

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