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Improved pFastBac™ donor plasmid vectors for higher protein production using the Bac-to-Bac® baculovirus expression vector system

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A B S T R A C T

The Autographa californica multiple nucleopolyhedrovirus (AcMNPV)-based Bac-to-Bac® expression system consists of a bacmid and five pFastBac™ donor transfer vectors. It has been widely used for eukaryotic gene expression in insect cells to elucidate gene function in biotechnology laboratories. The pFastBac™ vectors contain a 50 bp AcMNPV polyhedrin (*polh*) promoter and a 127 bp SV40 polyadenylation (pA) signal for cloning a gene of interest into the bacmid, resulting in unsolved lower gene expression levels than the wild type (wt) AcMNPV in insect cells. Therefore, the purpose of this research is to understand why the Bac-to-Bac system produces lower gene expression levels. Here, we determined that bacmids transposed with pFastBac™ vectors produced 3–4 fold lower levels of certain proteins than the wt AcMNPV. We found that an 80 bp *cis* element 147 bp upstream of the 50 bp *polh* promoter and a 134 bp *polh* pA signal are required in pFastBac™ to achieve bacmid protein expression levels equivalent to wt AcMNPV in High Five insect cells. Therefore, researchers currently using pFastBac™ vectors for protein expression can transfer their genes of interest into the improved vectors in this report to elevate protein expression yields in insect cells to reduce protein production costs.

1. Introduction

Insect-specific baculoviruses in the family *Baculoviridae* have circular, double-stranded, DNA genomes in the range of 88–180 kb (Herniou et al., 2012). Baculovirus research focuses on molecular and genetic studies, protein display as well as eukaryotic gene expression (Grabherr and Ernst, 2010; Passarelli and Miller, 1993; Rodems and Friesen, 1993; Smith et al., 1983). Of all the baculoviruses, Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the most studied, and it is the foundation of the baculovirus expression vector system (BEVS) (Hopkins et al., 2010). AcMNPV is preferred because it has the propensity to replicate efficiently in IPLB-Sf21-AE (Sf21), Sf9 (cloned from Sf21) and BTI-Tn-5B1-4 (High Five™) insect cells and can produce a high concentration or titer of budded virus (BV) (Cheng et al., 2013; Granados et al., 1994; Summers and Smith, 1987).

AcMNPV cell infection is accompanied by high levels of expression of a virus-encoded protein called polyhedrin, which forms large paracrystalline particles of 0.5–15 μm in diameter in the nuclei during late phase infection (Tanada and Haya, 1993). Production of these particles, formally known as polyhedra, requires large amounts of polyhedrin protein. This high level of protein is generated from a huge pool of mRNA produced under a very strong polyhedrin (*polh*) promoter. High-level *polh* promoter-mediated transcription requires 19 late expression factors (*lef*), one very late expression factor-1 (VLF-1), and a multifunctional protein (FP25K) (Cheng et al., 2013; Lu and Miller, 1995). Due to the high protein expression level mediated by the *polh* promoter in insect cells, AcMNPV has been used commercially to produce prophylactic vaccines, such as Cervarix® to fight against cervical cancer caused by human papillomavirus (HPV) and FluBlok® to reduce influenza virus infection in humans (Cox and Hashimoto, 2011; Harper, 2009).

The most widely used AcMNPV *polh* promoter-based BEVS in the biotech industry and research laboratories is the Bac-to-Bac system®, constructed in the late 1990's and marketed by Invitrogen (Carlsbad, CA) (Luckow et al., 1993). The Bac-to-Bac system® involves site-specific transposition between a clonal copy of the AcMNPV genome (bacmid)

Abbreviations: polh, polyhedrin; pA, polyadenylation; AcMNPV, Autographa californica multiple nucleopolyhedrovirus; HPV, human papillomavirus; UTR, untranslated region

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and a pFastBac™ donor plasmid to produce recombinant bacmid DNA in DH10Bac™ *Escherichia coli* cells with the aid of a helper plasmid. The helper plasmid expresses a transposase to transfer the gene of interest from the pFastBac™ donor plasmid to a specific site within the bacmid *in vivo*. The Bac-to-Bac® system eliminates the lengthy (up to 6 months) plaque-purification step required by the conventional homologous recombination method to produce the recombinant virus (Kitts et al., 1990; Smith et al., 1983). Due to the ease with which foreign genes can be cloned into the AcMNPV bacmid, the Bac-to-Bac™ system along with its five pFastBac vectors (pFastBac1, pFastBac Dual, and pFastBacHT-a, −b, −c) have become a powerhouse second only to the *E. coli* expression system for eukaryotic protein structure studies, as shown in the worldwide Protein Data Bank (Gabanyi and Berman, 2015).

It was reported that the Bac-to-Bac® system expresses lower protein yields than the conventional BEVS (Gomez-Sebastian et al., 2014). However, the elements at the *polh* locus that regulate protein expression yields are unknown to date. Therefore, the aim of this project is to identify these elements to improve protein production yields in insect cells.

In this report, we show that certain protein expression levels using the Bac-to-Bac® system are not as high as the wild type (wt) AcMNPV in certain insect cell lines. The donor plasmid vectors such as pFastBac™1 and pFastBac™ Dual lack an 80 bp *cis* DNA element and contain a 127 bp SV40 polyadenylation (pA) signal. When the 80 bp *cis* DNA element was inserted upstream of the 50 bp *polh* promoter and the SV40 pA was replaced with an AcMNPV *polh* pA signal in pFastBac™1 and pFastBac™Dual, certain protein expression levels equaled that of the wt AcMNPV in High Five cells using the Bac-to-Bac® system.

2. Materials and methods

2.1. Cell lines, viruses and plasmids

Insect cell lines used in this project included High Five, Sf21 and Sf9 cells, all obtained from Invitrogen. The wild type (wt) virus used in this study was AcP3, a plaque-purified AcMNPV E2 strain originally received from Dr. Max Summers of Texas A & M University (Cheng et al., 2013). Plasmids and bacterial strains used were pFastBac™1, pFastBac™Dual, and the host bacterial strain DH10Bac, obtained from the Bac-to-Bac® system kit (Invitrogen).

2.2. Modification of pFastBac™1 to produce improved donor transfer vector pFastBac-M1

Although the exact reason for the poorer protein expression yield of the Bac-to-Bac® system compared to the wt AcMNPV was unknown, we first amplified a 1.5 kb DNA fragment by PCR using a forward primer AcPolh-F-XbaI and reverse primer AcPolh-R-XhoI and cloned it into the pGEM-T Easy vector (Promega, Madison WI) to produce pGEM-PolhE (Table 1). This 1.5 kb fragment contained the *polh* ORF with 319 bp of DNA sequence upstream of the *polh* ORF start codon ATG. The 319 DNA sequence included the 50 bp *polh* promoter and additional upstream sequences. The 1.5 kb fragment also included the *polh* downstream untranslated region (UTR) containing a 472 bp *polh* polyadenylation signal (pA) between nucleotides (ntd) 739–1211 (Figs. 1 A1 and 2).

To evaluate the effect of this 1.5 kb fragment on polyhedrin protein expression using the Bac-to-Bac® system, the 1.5 kb fragment from pGEM-PolhE was retrieved with restriction endonucleases (REN) XbaI and XhoI (NEB, Ipswich, MA) and inserted into these sites in pFastBac™1 to generate a clone (pAcBac-PolhE) (Fig. 1B; Fig. 2). Competent DH10Bac cells were transformed with pAcBac-PolhE and recombinant bacmid clones were screened and selected using X-gal and IPTG on antibiotic plates, following conditions recommended by Invitrogen. One confirmed recombinant bacmid with the 1.5 kb *polh* fragment was used to transfect High Five cells to generate AcBac-PolhE budded virus (BV).

Table 1
A list of primers used in this study.

Primer names	Primer sequences (restriction enzyme sites underlined)
AcPolh-F-XbaI	5′-tctagagcatagtacgcagcttcttc-3′
AcPolh-R-XhoI	5′-ctcgagtaacacgcccgatgttaaa–3′
AcPolh-F-EcoRI	5′-gaattcatgccggattattcatacc-3′
Hind-F	5′-ataaagctaggacatatttaacatcgggcgtgttag-3′
Hind-R	5′-atgtcctagctttatttaacgtgtttacgtcgagtc-3′
Polh-F1-HindIII	5′ −cccaagcttcttgtagcagcaatctag-3′
Polh-R-BamH1	5′-cggatccaatatttataggttttttattacaaaactg-3′
Promoter-R1	5′- gttaatccggtgctgc-3′
Promoter-R2	5′-aaaagggaggtgaactg-3′
Promoter-R3	5′- gtctcattacaatggctg-3′
Promoter-R4	5′-ctatatattgatagacatttccag-5′
promoter-F	5′-gatatcatggagataattaaaatg −3′
CisF1	5′-gtagcatagtacgcagcttctt-3′
Polh-R-BamH1	5′-cccggatccaatatttataggtttttttattacaaaactg-3′
Ac-Polh-F-EcoRI	5′-gaattcatgccggattattcatacc-3′
Ac-Polh-R-XbaI	5′ −tctagattaatacgccggaccag-3
HPV16 L1-F1-XbaI	5′-tctagaatggaggtgactttatttacatc-3′
HPV16 L1-R1-HindIII	5′-aagcttttacagcttacgtttttgcg-3′
AcpolhF	5′-cccagatctatgccggattattcatac-3′
AcpolhR1	5′-ggggtcgacgataacggcacctaaat-3′

The AcBac-PolhE construct had two *polh* promoters; one from the parental pFastBac1 vector and one from the upstream sequences of the 1.5 kb DNA fragment (Fig. 2). Also, AcBac-PolhE had two pAs; the SV40 pA from the pFastBac1 vector and the *polh* pA from the 1.5 kb DNA fragment (Fig. 2). To delineate the functionality of the 1.5 kb insert in AcBac-PolhE, the vector *polh* promoter and SV40 pA of pAcBac-PolhE were deleted. The vector *polh* promoter was deleted by digestion of pAcBac-PolhE with BstZ17I and XbaI, followed by Klenow enzyme treatment and self-ligation with T4 DNA ligase (NEB) to generate the plasmid pAcBac-PolhED. The SV40 pA was deleted by digestion of pAcBac-PolhED with XhoI and AvrII, followed by Klenow enzyme treatment and self-ligation with T4 DNA ligase to generate plasmid pAcBac-PolhED-XX. To use the unique HindIII site of the donor vector for cloning genes, the HindIII site in the UTR of *polh* was mutated from AAGCTT to AAGCTA by site-directed mutagenesis using the primer pair Hind-F and Hind-R (Table 1) and the QuikChange II Site–Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). This resulted in the generation of the plasmid pAcBac-PolhED-XXH (Fig. 2), which was necessary for the subsequent steps of engineering pFastBac1-M1.

Inverse PCR was used to produce pFastBac-M1. A pair of primers (Polh-F1-HindIII and Polh-R-BamH1) using pAcBac-PolhED-XXH DNA as a template and the high fidelity *pfu* enzyme (Agilent Technologies) produced a linear DNA fragment that was digested with HindIII and BamHI (Table 1). The digested linear DNA fragment was then ligated with T4 DNA ligase into the multiple cloning site (MCS) fragment retrieved from pFastBac™1 digested with HindIII and BamH1, thus producing pFastBac-M1 (Fig. 1B).

To determine if all the upstream sequences of the *polh* promoter were required for the improved protein expression yield of pFastBac-M1, four reverse primers (Promoter-R1, −R2, −R3 and −R4, Table 1) were designed to map the 240 bp region upstream of the promoter (Fig. 1A2; Table 1). Each of the four reverse primers was paired with primer promoter-F in inverse PCR, in order to delete a defined length of DNA sequence in the 240 bp region immediately upstream of the *polh* promoter, using pAcBac-PolhED-XXH DNA as a template with the *pfu* DNA polymerase. The promoter-F and promoter-R3 reaction ultimately produced the clone pAcBac-MR3-Polh, which was missing 144 bp (ntd −240 to −96, Fig. 1A3) of the 240 bp upstream region but maintained the rest of the plasmid sequences, including an 80 bp DNA sequence upstream of the 50 bp *polh* promoter and the *polh* pA (Fig. 2). Competent DH10Bac™ cells were transformed with pAcBac-MR3-Polh DNA to generate AcBac-MR3-Polh. This bacmid DNA was transfected into High Five cells to produce BV for infection of High Five cells, which were

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