Development of a rapid and efficient BmNPV baculovirus expression system for application in mulberry silkworm, *Bombyx mori*

Cuiping Cao[1], Xiaofeng Wu[1,*], Na Zhao[1], Huipeng Yao[1], Xingmeng Lu[1] and Yeping Tan[2]

[1]Lab of Bioresource and Biotechnology, College of Animal Sciences, Zhejiang University, Huajiachi Campus, Hangzhou 310 029, China
[2]Department of Entomology, University of California, Riverside, CA 92521, USA

Silkworm–baculovirus gene expression system is one of the most powerful eukaryotic expression systems. However, due to very low recombination frequency, the traditional method to construct and obtain pure recombinant baculovirus requires plaque assay that is time-consuming and also utilizes skillful techniques. In order to overcome this disadvantage, a rapid BmNPV expression system applicable to silkworm was constructed based on the working principle of AcMNPV Bac-to-Bac system. A large 8.6 kb fragment containing the low-copy-number mini-F replicon, a kanamycin resistance marker and a segment of DNA encoding the *lac*Zα peptide from the AcMNPV bacmid, was cloned into polyhedrin locus of BmNPV genome to replace the polyhedrin gene. This recombinant, designated as BmBacmid, was transformed into *Escherichia coli* DH10β strain, in which a helper plasmid encoding the transposase was already transformed. We designated the DH10β strain containing BmBacmid and helper as DH10BmBac. With this bacterium, the recombinant baculovirus can be rapidly and easily generated through gene transposition. This system has an advantage of high recombination frequency, simple manipulation, high-efficiency and is time-saving. This approach permits large potential value in recombinant protein production using silkworm as a 'biofactory' in the future biotechnological industry and can become a powerful tool for structural and functional analysis of protein in post-genomic era.

Keywords: Baculovirus, Bac-to-Bac system, expression system, *Mulberry* silkworm.

THE baculovirus expression vector system (BEVS) uses recombinant baculovirus harbouring a foreign gene of interest to infect the host insect or its cultured cells to produce the recombinant protein. It has proved to be one of the most powerful eukaryotic expression systems available[1,2]. Several unique features of the BEVS have made it the system of choice for many applications. Proteins expressed in BEVS are, in most cases, soluble and functionally active. Compared to other higher eukaryotic expression systems, the most distinguishing feature of the BEVS is its potential to achieve high levels of expression of a cloned gene. Currently there are two types of baculovirus-based expression systems used for the production of recombinant proteins, the *Autographa californica* nucleopolyhedrovirus (AcMNPV) which infects Sf9/Sf21 cells and *Trichoplusia ni* larvae, and the *Bombyx mori* nucleopolyhedrovirus (BmNPV) that infects silkworm cells and larvae. The AcMNPV has been extensively used for high-level expression of foreign genes, but has limited capability of post-translational modifications. Recombinant proteins can be produced easily in silkworm larvae and pupae using the BmNPV vector system. Since silkworm rearing is routinely practised for silk production in some Asian countries like China, India and Japan, a part of the silkworm stock can be used to produce biomolecules other than silk and more sophisticated tissue-culture methodologies can be dispensed with on an industrial scale[3]. Furthermore, the large body of silkworm allows efficient production of proteins and vaccines in gram scale at low costs. This would have the potential of supplying global demand at costs that would be economically feasible for most developing countries.

The construction of recombinant viruses, however, is a time-consuming step that restricts consideration of the technology for throughput developments[4]. The traditional method of recombinant baculovirus construction involves two steps. First, the foreign gene of interest is cloned into the transfer vector, and subsequently co-transfected with virus DNA into cultured cells where homologous recombination takes place. The recombination frequency by this method is as low as 0.1 to 1%. The recombinant progeny virus can be improved up to 30 to 80% using parent virus that is linearized at one or more unique sites located near the target site for insertion of the foreign gene into the baculovirus genome[5,6]. However, this method is still tedious and needs skillful handling for sequential plaque assays to isolate recombinant baculovirus from non-recombinant ones; thus making it difficult for a beginner to use this system. Moreover, several rounds of plaque assay may result in the contamination of progeny virus after transfecting the plasmid and viral DNAs into insect cells. To overcome this problem, a big innovation of generating the recombinant baculovirus was achieved by Luckow and coworkers[7–9] utilizing bacterial transposon to construct Bac-to-Bac system in AcMNPV. This system is based on site-specific transposition of an expression cassette through bacterial transposon Tn7 into baculovirus shuttle vector propagated in *Escherchia coli* (Figure 1 *a*). The major advantage of this system is that the recombinant virus can be generated more efficiently and rapidly. It is well established that the baculovirus strains are highly species-specific and the host range of most widely used AcMNPV is restricted to *Spodoptera frugiperda* cell lines and *T. ni* larvae. As the bacmid generated by AcMNPV does not work for silkworm, it is essential to generate a system which readily infects *B. mori*. Keeping this in view we developed an effi-

*For correspondence. (e-mail: wuxiaofeng@zju.edu.cn)

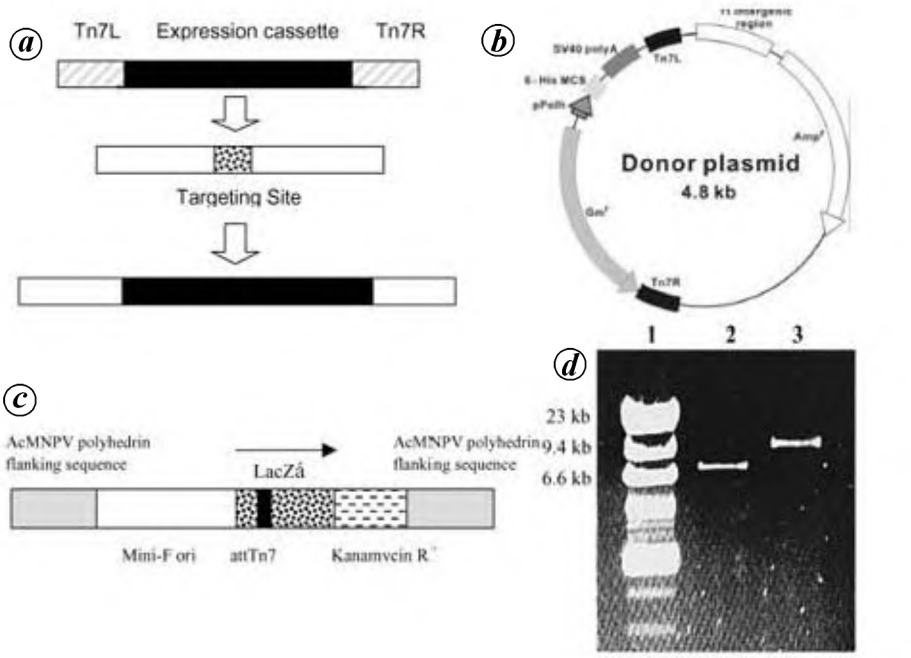

Figure 1. *a*, Working principle of gene transposition by bacterial transposon Tn7. *b*, Donor plasmid compatible for Bac-to-Bac baculovirus expression system. The most distinguished characteristics of this vector is the presence of left and right arm of bacterial transposon Tn7, as well as an expression cassette containing baculovirus-specific promoter, a multiple cloning site. *c*, Schematic representation of large 8.6 kb fragment in bacmid of AcMNPV system. It contains the low-copy-number mini-F replicon, a kanamycin resistance marker, a segment of DNA encoding the *lac*Zα peptide and targeting site for bacterial transposon. *d*, Cloning of 8.6 kb (lane 2) and isolation of helper plasmid (lane 3); DNA marker is presented in lane 1.

cient BmNPV Bac-to-Bac system applicable to silkworm *B. mori* and its cell lines.

The Bac-to-Bac baculovirus expression system was purchased from Invitrogen (San Diego, CA, USA). The major components of this system include donor plasmids (Figure 1 *b*) that allow generation of an expression construct containing the gene of interest, where expression of the gene of interest is controlled by a baculovirus-specific promoter, an *E. coli* host strain, DH10Bac™ (Max efficiency® DH10Bac™ competent cells), that contains a bacmid and a helper plasmid, and allows generation of a recombinant bacmid following transposition of the donor expression construct. Foetal calf serum (FCS), culture medium TC-100 for cultured cells were products of GibcoBRL (Gaitherburg, MD, USA). The baculovirus transfer vector pBlueBacHis and cellfectin reagent for transfection were also purchased from Invitrogen.

Spodoptera frugiperda insect cells (Sf9) and *B. mori* BmN cells were maintained at 27°C in TC-100 medium supplemented with 10% fetal calf serum. BmNPV was stored in our laboratory. A hybrid strain of silkworm (commercial name: *Jingsong* × *Haoyue*) was used for infection test. The larvae were reared on mulberry leaves at 23–25°C.

A DNA fragment containing the low-copy-number mini-F replicon, a kanamycin resistance marker, a segment of DNA encoding the *lac*Zα peptide and targeting site for bacterial transposon (inserted into the N-terminus of *lac*Zα gene, is a short segment containing the attachment site for the bacterial transposon Tn7, i.e. mini-*att*Tn7 that does not disrupt the reading frame of the *lac*Zα peptide; Figure 1 *c*), was cloned from the AcMNPV bacmid extracted from the Max efficiency® DH10BacP™ cells by PCR. The primers were designed as sense primer – 5′-UGCCACGTTGTG TCTCAAAATC-3′ and antisense primer – 5′-UUTA GCTTCATCGTGTCGGGTTT-3′. The PCR reaction was carried out by the following conditions using Expand Long Template PCR System (Roche): denature template for 2 min at 94°C; 10 cycles for the following steps, denaturation at 94°C for 10 s, annealing at 65°C for 30 s, elongation at 68°C for 8 min; 20 cycles for the following steps, denaturation at 94°C for 10 s, annealing at 65°C for 30 s, elongation at 68°C for 8 min + cycle elongation for further yield of 20 s for each cycle; finally elongation at 68°C for 7 min. The PCR product was identified by agarose gel electrophoresis (Figure 1 *d*).

The baculovirus transfer vector pBlueBacHisa (Figure 2) was digested with *Hin*dIII and *Xba*I, resulting in a linearized vector with recombination sequences flanking the polyhedrin gene. This vector and the above 8.6 kb fragment were further treated with Klenow Fragment (Takara, Japan) to form blunt ends, and ligated to produce a tool vector for the generation of BmNPV bacmid. We named this vector as pBlueBac-8.6.

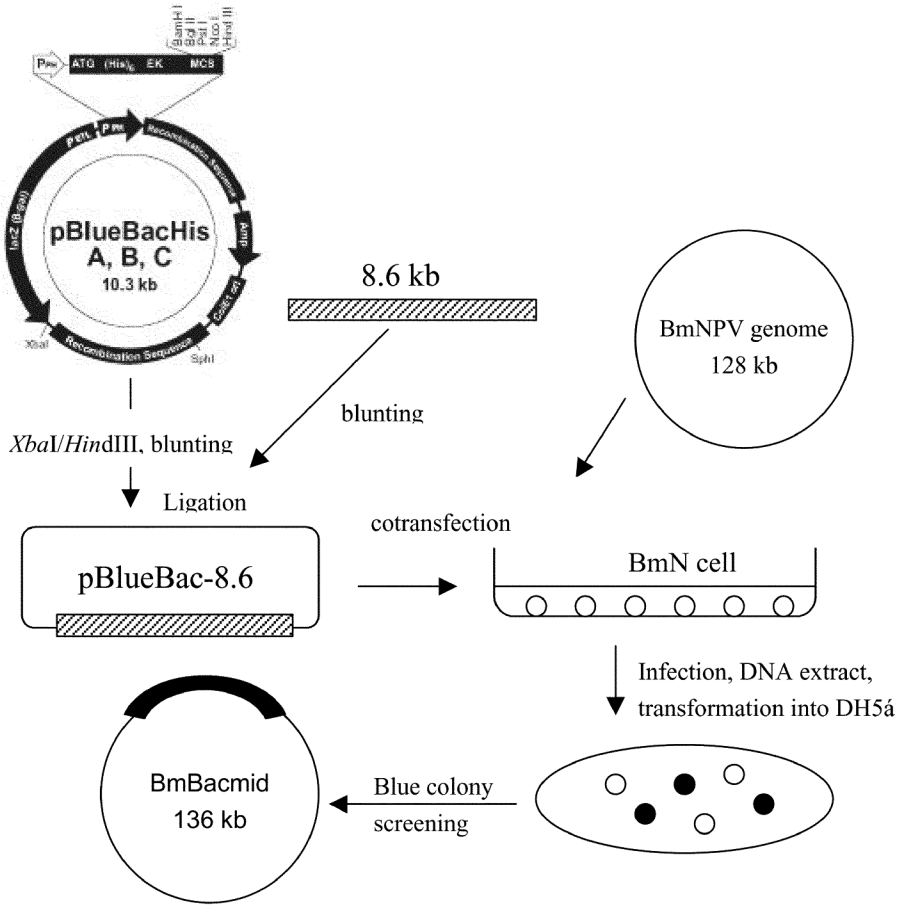

Figure 2. Flow chart of strategy to generate a BmNPV bacmid. Details described in text.

The flow chart of generating the BmNPV bacmid is shown in Figure 2. The BmNPV genome was extracted from infected BmN cells using DNA extract kit (Stratagene). The pBuleBac-8.6 was co-transfected with BmNPV DNA into BmN cells, where homologous recombination will occur as the sequences flanking the polyhedrin gene of BmNPV and AcMNPV share high homology. After 5 days, the medium was collected and centrifuged, and subsequently the supernatant was inoculated to BmN cells again. The above-mentioned procedure was repeated several times in order to remove the plasmid pBlueBac-8.6 DNA completely. The last infected BmN cells were collected and DNA was extracted. This DNA was transformed into DH5α competent cells by electroporation and spread on the LB plate containing kanamycin (50 μg/ml) smeared with X-gal in advance. Within the following 24–48 h, the blue and bigger colonies were selected and cultured overnight, and DNA was extracted according to the protocol for isolating large plasmids (>100 kb) (Invitrogen Instruction Manual of Bac-to-Bac systems). The large plasmid thus obtained was further identified by PCR reaction to confirm the insert of 8.6 kb fragment into BmNPV genome, and was named as BmBacmid. The BmBacmid was transformed into the competent cells DH10β, in which a helper plasmid (13.2 kb, Figure 1 *d*) encoding the transposase was already transformed, which could confer resistance to tetracycline and encode the transposase. The transformants were screened by growth on the LB agar plate containing kanamycin (50 μg/ml), tetracycline (7 μg/ml), X-gal (100 μg/ml) and IPTG (40 μg/ml). The blue colonies were cultured and prepared into competent cells, which were named as DH10BmBacmid. In addition to the donor plasmids which are already commercially available, we developed a novel Bac-to-Bac baculovirus expression system to produce recombinant BmNPV, which can be used to infect silkworm and its cell lines. We named this as BmNPV Bac-to-Bac baculovirus expression system.

To test the efficiency of the above constructed BmNPV Bac-to-Bac system in silkworm, two popular reporter genes, *EGFP* and *LacZ* were used for generation of recombinant baculovirus and expression. The 720 bp of *EGFP* was cloned in *Eco*RI and *Spe*I sites of donor plasmid pFastBacHTc. The 3090 bp of *LacZ* gene was first cut from the vector pRSET-LacZ (Invitrogen) and then cloned into the *Bam*HI and *Hin*dIII sites of pFastBacHTa. Approximately 1 ng of the obtained recombinant donor plasmid was transformed into 100 μl of the competent cells, DH10BmBacmid. The mixture was incubated at 37°C for

4 h for the occurrence of transposition. Then the cells were serially diluted using SOC medium and spread evenly on plates containing kanamycin, tetracycline and X-gal. After 48 h of incubation at 37°C the white colonies were picked and cultured in medium containing kanamycin overnight. The bacmid DNA was subsequently extracted and transfected into BmN cells to generate the recombinant baculoviruses. After 5 days, the medium supernatant was collected and used to infect silkworm larvae.

The key step in developing BmNPV Bac-to-Bac baculovirus expression vector system is the insertion of large 8.6 kb fragment directly into BmNPV. As the BmNPV genome is too large (128, 413 bp), it is impossible to directly insert the 8.6 kb fragment into BmNPV. For this reason, we first cloned this fragment to baculovirus transfer vector and used it as a tool for homologous recombination with BmNPV genome in BmN cells. The cultured cells, after co-transfection will contain a mixture of recombinant BmNPV, wild-type BmNPV and pBlueBac-8.6. Since BmBacmid as well as pBlueBac-8.6 can form blue colour on LB plates containing X-gal, it is difficult to identify them. Hence, it is necessary to remove pBlueBac-8.6 before transforming the recombinant DNA from infected cells to *E. coli*. In order to purify recombinant virus away from pBlueBac-8.6, the viruses collected from the culture medium after cotransfection were used to inoculate BmN cells and this step was repeated several times. Finally DNA was extracted from the bacterial cells of the blue colony, and the baculovirus DNA was confirmed by agarose gel (0.5%) electrophoresis. Further, the large DNA was transfected into BmN cells to generate virus, and after 5 days the supernatant was collected and used to infect both BmN cells and silkworm larvae to test its infection capacity. It was observed that both the BmN cells and silkworm larvae were well infected. Moreover, no polyhedrin was detected in infected cultured cells or larval haemolymph. Hence, these results indicate that this virus is the recombinant BmNPV baculovirus and the large DNA isolated from the blue colony is actually the recombinant baculovirus DNA containing the 8.6 kb fragment. Thus, we have successfully generated and isolated the BmBacmid. The BmBacmid was subsequently transformed into the DH10β competent cells, followed by transformation of a helper plasmid.

At present, small-size *EGFP* and large *LacZ* are extensively used as reporter genes. In our experiment we used these genes to generate the recombinant baculoviruses using the above constructed system to observe its working efficiency. The first step is to subclone the gene of interest into the donor plasmid pFastBacHT (4.8 kb; Figure 3 *a*), and this operation is common and easy. The second step is to transform the recombinant donor into DH10 BmBacmid competent cells for gene transposition, followed by colony selection according to the colour on LB plates containing antibacterial agents and X-gal. This step usually takes 2–3 days. The final step is to extract the large bacmid DNA from the bacteria of white colonies and transfect them into BmN cell to produce the recombinant baculovirus, and it takes 4–5 days. By the above methods, the recombinant baculovirus harbouring *EGFP* and *LacZ* gene respectively, was successfully generated and only took 7–8 days, no matter what its size. Hence, this Bac-to-Bac system for silkworm is time-saving and highly efficient. Furthermore, both *EGFP* and LacZ genes showed high expression by initiating blue light and blue colour formation respectively (Figure 3 *b*).

In China, sericulture has been practised for several centuries for silk production, where the mulberry silkworm *B. mori* has been used as a tool to convert leaf protein into silk protein. Development in the fields of molecular biology and biotechnology opened new areas of research in silkworm biology. At present silkworm is being utilized as a powerful eukaryotic model in basic studies and as a bioreactor for foreign protein production using BEVS in applied research. Numerous reports are available addressing silkworm as an efficient biofactory for the production of heterologous proteins. Recently, Japanese scientists developed transgenic silkworm using baculovirus and piggybac transposon[10,11]. The transgenic silkworm could spin human skin protein collagen along with silk fibre. A variety of human proteins are also produced using the BmNPV vector system. In our own laboratory, we successfully produced large amounts of foreign proteins in silkworm, including human FGFs, VEGF and lactoferrin[12,13]. Since silkworms are distantly related to humans, proteins produced by them would be safer compared to other sources. The baculovirus expression system has already gained prominence in various research areas such as pharmaceuticals, where rapid expression of recombinant proteins is highly essential for structure determination. The success of this system can be attributed to its capacity for insertion, high yield of recombinant protein and probability of success compared to protein expression in *E. coli*. However, AcMNPV is the only BEVS that has been widely accepted. The major drawback of this system is the expense involved in generating cell lines and their maintenance under appropriate conditions which is laborious. The exploitation of BmNPV using silkworm as a host has some outstanding advantages over AcMNPV, such as high efficient production due to the large body of silkworm, low cost of production and easy rearing.

However, construction of recombinant baculovirus needs skillful techniques and plaque assay is essential for isolating pure recombinants. The procedure of construction and purification of a recombinant baculovirus using the plaque assay usually takes 2–3 months or longer resulting in a bottleneck in the application of this system. In this study, we have developed a BmNPV Bac-to-Bac system applicable to silkworm.

The silkworm Bac-to-Bac baculovirus expression system provides the following advantages over the traditional method for homologous recombination: (i) It requires

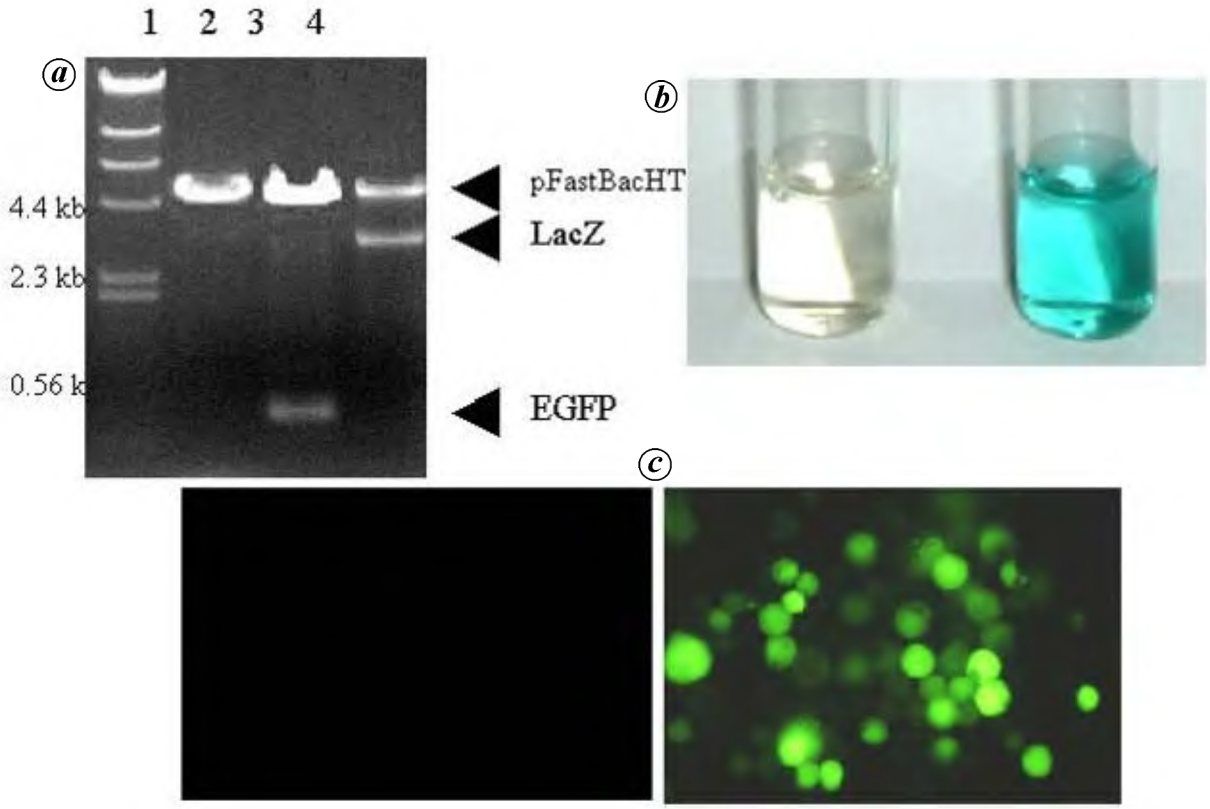

Figure 3. ***a***, Subcloning of the *EGFP* (720 bp, lane 3) and *LacZ* (3090 bp, lane 4) into donor plasmid vector pFastBacHT, identified by restriction enzyme digestion. ***b***, Expression of *LacZ* gene by recombinant baculovirus in BmN cells. The medium supernatant of 72 postinfection was collected and X-gal, its substrate for galactosidase was added for colour reaction. ***c***, Expression of *EGFP* gene in BmN cells. The medium supernatant of 72 postinfection was collected and initiated by blue light, and observed under reverse microscope (400×), the control infected by wild BmNPV is also presented (left).

less than two weeks to identify and purify a recombinant baculovirus compared to the 2–3 weeks required to generate a recombinant baculovirus using homologous recombination. (ii) It reduces the need for multiple rounds of plaque purification as the recombinant virus DNA isolated from selected colonies is not mixed with parental, non-recombinant virus. (iii) It permits rapid and simultaneous isolation of multiple recombinant baculoviruses, and is suited for the expression of protein variants for structure/function studies.

With this rapid and high-efficient BmNPV Bac-to-Bac system, it is anticipated that BEVS will be extensively applied in developing countries where silkworm is reared continually. Moreover, silkworm can be utilized more efficiently for production of recombinant proteins, thus favouring the development of biotechnological industry. Besides, it can also be used as a powerful tool for structural and functional analysis of proteins in post-genomic era.

1. O'Reilly, D., Miller, L. K. and Luckow, V. A., *Baculovirus Expression Vectors: A Laboratory Manual*, W.H. Freeman and Company, New York, 1992.
2. Kidd, I. M. and Emery, V. C., The use of baculoviruses as expression vectors. *Appl. Biochem. Biotechnol.*, 1993, **42**, 137–159.
3. Acharya, A., Sriram, S., Sehrawat, S., Rahman, M., Sehgal, D. and Gopinathan, K. P., *Bombyx mori* nucleopolyhedrovirus: Molecular biology and biotechnological applications for large-scale synthesis of recombinant proteins. *Curr. Sci.*, 2002, **83**, 455–465.
4. Zhao, Y., Chapman, D. A. and Jones, I. M., Improving baculovirus recombination. *Nucleic Acids Res.*, 2003, **31**, E6.
5. Kitts, P. A., Ayres, M. D. and Possee, R. D., Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Res.*, 1990, **18**, 5667–5672.
6. Hartig, P. C. and Cardon, M. C., Rapid efficient production of baculovirus expression vectors. *J. Virol. Methods*, 1992, **38**, 61–70.
7. Luckow, V. A., Lee, C. S., Barry, G. F. and Olins, P. O., Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.*, 1993, **67**, 4566–4579.
8. Anderson, D., Harris, R., Polayes, D., Ciccarone, V., Donahue, R., Gerard, G. and Jessee, J., Rapid generation of recombinant baculoviruses and expression of foreign genes using the Bac-to-Bac® baculovirus expression system. *Focus*, 1956, **17**, 53–58.
9. Ciccarone, V. C., Polayes, D. and Luckow, V. A., *Generation of Recombinant Baculovirus DNA in E. coli Using Baculovirus Shuttle Vector* (ed. Reischt, U.), Humana Press, Totowa, NJ, 1997, vol. 13.

10. Yamao, M. *et al.*, Gene targeting in the silkworm by use of a baculovirus. *Genes Dev.*, 1999, **13**, 511–516.
11. Tomita, M. *et al.*, Transgenic silkworms produce recombinant human type III procollagen in cocoons. *Nature Biotechnol.*, 2003, **21**, 52–56.
12. Xiaofeng Wu *et al.*, High-level expression of human acidic and basic fibroblast growth factors in the silkworm, *Bombyx mori* L. using recombinant baculovirus. *Protein Expres. Purif.*, 2001, **21**, 192–200.
13. Xiaofeng Wu, Zhaozheng Yin, Cuiping Cao, Ling Huang, Xingmeng Lu, Jianxin LiuB and Weizheng Cui, Expression of human VEGF165 in silkworm *Bombyx mori* by using a recombinant baculovirus and its bioactivity assay. *J. Biotechnol.*, 2004, **111**, 253–261.

ACKNOWLEDGEMENTS. This work was supported by National Basic Research Programme (2005CB121003) and Provincial Key International Cooperation Programme of Zhejiang (2006C24015). We sincerely thank Dr K. Gokulamma, Department of Studies in Sericultural Science, Manasagangotri, University of Mysore, India for a critical reading of this manuscript.

Received 9 March 2006; revised accepted 10 August 2006

Microbial DNA extraction from samples of varied origin

S. Ray Chaudhuri*, A. K. Pattanayak and A. R. Thakur

West Bengal University of Technology, BF 142, Sector I, Salt Lake, Kolkata 700 064, India

The impact of four different soil DNA extraction methods on the quantity and quality of isolated community DNA was evaluated using agarose gel electrophoresis, DNA spectrum study and PCR-based 16S ribosomal DNA analysis. The modified direct lysis method was optimized for environmental water samples (wastewater fed-fisheries and raw liquid sewage canal) as well as diagnostic samples like urine from humans, thus opening up a new arena for fast culture-independent detection of causative pathogens in pathological manifestations like urinary tract infections. The same method was independently found to be effective for isolation of genomic DNA from both Gram-positive and Gram-negative bacteria. Thus a uniform method of DNA extraction from environmental samples (different types of soil and water), pathological samples as well as varied kinds of bacteria was obtained.

Keywords: Community DNA, DNA extraction, microbial biodiversity, urine microbial analysis.

*For correspondence. (e-mail: shaon_raychaudhuri@yahoo.co.in)

THE microbial diversity studies conducted in complex ecosystems, such as soil and sewage have often been found to be biased. Essentially this has been due to the inability to culture many microorganisms[1,2]. Reports indicate that only 1 to 4% of the microbes can be cultivated under the standard laboratory conditions[3,4]. In the past decade, applications of new methods based primarily on soil-extracted nucleic acids have provided an alternative to classical culture based microbiological methods, providing unique insight into the composition richness and structure of microbial communities[5]. In case of molecular analysis of microbial communities, the bottleneck remains the isolation of pure community DNA in reasonable quantity. Steps for removing contamination of humic acid and fulvic acid are both complicated and expensive[6]. No universal method for extraction of community DNA from samples of varied origin is available. In the past decade comparative studies have been performed to analyse the efficiency of methods for extraction and purification of soil DNA, revealing that these methods suffer from low efficiency, mainly due to incomplete cell lysis and DNA adsorption to soil particles[7,8]. However, the impact of the extraction method on the outcome of indigenous microbial community analysis has not been clearly established[8]. In recent years DNA analysis has been extensively used for taxonomy as well as diagnostics[9]. PCR-based microbial detection of pathogens in case of meningitis, septicemia, tuberculosis and genitourinary tract infection has already been reported[10–14]. Thus obtaining a viable method for community DNA extraction from samples of varied origin (with minor modifications) and characteristics has become an essential requirement for these studies. This study shows how the modified direct lysis method can actually give good yield of DNA from a large number of varied sources for doing molecular biology work.

Four different methods of DNA extraction[15] with minor modifications were tried with soil samples. The direct extraction method was similar to extraction by bead beating, except that the cell lysis was obtained by repeated inversion of the suspended sample in extraction buffer for 10 min at room temperatures. In each case, 8 ml of extraction buffer was used for 4 g of soil. DNA extractions by sonication as well as enzymatic lysis were followed according to the method of Yeates *et al.*[15]. A modified indirect method of DNA isolation and purification was followed[16].

For DNA extraction from water samples like raw sewage canal and wastewater fed fisheries, the direct extraction method with modification was used. Here the water was centrifuged to pellet down the cells at 10,000 *g* for 10 min and washed thrice with buffer A [50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0)] before lysis. For isolation of DNA from urine, the direct extraction method was used with further modifications as mentioned below. The urine was centrifuged at 10,000 *g* for 10 min. The supernatant was discarded and the pellet was washed with phosphate buffer saline before proceeding for lysis. This is to ensure