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In vitro production of *Helicoverpa* baculovirus biopesticides—Automated selection of insect cell clones for manufacturing and systems biology studies

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A B S T R A C T

Baculovirus pesticides are increasingly being used as effective biological control agents against caterpillar pests worldwide. Increasing occlusion body (OB) yields per cell in culture is the main challenge to enable commercialization of in vitro production of baculovirus pesticides. Isolating clones from a heterogeneous cell population may allow development of a high virus producing cell clone. To date, the selection of insect clones has been based mainly on laborious cell serial dilution methods which create few viable clones. This work used an automated robotic clone picking system to establish over 250 insect clones of a *Helicoverpa zea* cell population to be screened for virus production. However, the higher producing clones only produced 10–30% higher OB yields than the original cell population. This study suggested that unless screening of thousands of clones is performed, obtaining a 2-fold increase in OB/cell yield compared to the parent population is unlikely. Nevertheless, it creates pure clones for manufacturing. In addition, two clones that were at least 2–3 times different in OB yields were isolated. Hence, this method can create a high contrast system (OB/cell yield basis), for comparative studies using a systems biology approach, which should inform a more targeted approach to engineer genetically a production cell line.

1. Introduction

Among 15 families of viruses known to infect insects, the virus strains in the Baculoviridae family have been most widely used as biopesticides to control several of the world's most devastating caterpillar pests (Szewczyk et al., 2009). Increasing chemical pesticide resistance as well as demands for more environmentally friendly options open up opportunities for baculovirus biopesticides (Jacobson et al., 2009). An occlusion body (OB), which occludes and maintains virions inside a polyhedral coat, is the active ingredient of baculovirus biopesticides. In-vitro production of OBs in a bioreactor offers a large scale production capacity, high product purity, and less labour costs compared with the traditional production methods which use infected caterpillars (Szewczyk et al., 2006). One of the main challenges to commercialize in vitro produced baculovirus pesticides is the low OB/cell yields normally observed in culture (Lua and Reid, 2005). Obviously, one way to improve virus yields is to improve the cell specific OB yield of the host cell employed following infection. To do this, two approaches are possible, namely a traditional approach of selecting monoclonal cell lines and screening for high producers. Alternatively, a more rational approach, of using modern systems biology tools to investigate host cell/virus interactions and then to use knowledge from this approach to better engineer genetically the insect host to improve yields of an existing clone in a targeted manner, is possible.

Cell lines are important for production of various biotechnology based products (Zhang et al., 2008; Smagghe et al., 2009; Popham et al., 2010). Selecting pure clones from heterogeneous cell populations can produce clones that display significant differences in yields of various biological products (Lindgren et al., 2009). Insect cell lines are generally heterogeneous populations, which are developed from minced tissues, most commonly from ovarian tissue (Pan et al., 2009; Wen et al., 2009). Lenz et al. (1991) estimated that the HzAM1 heterozygous population used in our laboratory had only $75 \pm 10\%$ of the cells producing OB. In other species such as *Spodoptera exigua* or *Clostera anachoreta*, two cell lines originated from the same species or even the same tissue can have different OB yields (Wen et al., 2009). The selection of high producing clones is important to increase production yield and stability. In addition, clone screening can isolate two clones highly different in OB yields, which can be useful for comparative studies using a systems biology approach. Advances in systems biology tools allow genome scale analysis which may inform engineering approaches for increasing production (O'Callaghan and James, 2008). Transcriptomics to compare high vs. low yielding clones is being studied to reveal differentially expressed genes in mammalian culture systems (Nissom

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et al., 2006). A current transcriptome sequencing project under way in this laboratory will allow the genome wide expression analysis of virus infected clones to identify gene targets for engineering insect cell lines to improve OB yields.

For baculovirus-insect cell systems, research on host cell lines has been limited compared with research on virus expression vectors. Common insect monoclonal cell lines such as *Trichoplusia ni* BTI-Tn-5B1-4 (High-Five) (Granados et al., 1994), and Sf-9 cells (Vaughn et al., 1977), were isolated by traditional limiting dilution methods. These methods can only create a small number of clones, mostly from 1 to 12 clones (Browne and Al-Rubeai, 2007; Zhang et al., 2009) and require a labour intensive workflow (Sleiman et al., 2008). Automation for cell line development of insect cell lines has not been developed as much as is the case of mammalian cell lines, where flow cytometry and automated cell picking systems are increasingly used for accelerating cell line development processes (Sleiman et al., 2008; Lindgren et al., 2009).

This study aims to isolate high virus producing clones by establishing and screening a large number of clones. For the first time, an automated robotic clone picking system was applied for insect cell line development to create a large number of clones for screening for virus production capacity. The screening process adapted high throughput methods such as flow cytometry for fast counting of the cells in batch culture processes, total protein assays for simultaneous estimation of cell biomass, flow cytometry for multiple cell size measurements, and the application of Inter simple sequence repeat (ISSR) markers for differentiating clones genetically.

The primary motivation for this work was the need to improve OB yields in culture if baculoviruses are to be successfully commercialized globally to combat insect pests. To date with the *Helicoverpa armigera* virus/*Helicoverpa zea* cell system, it is possible to produce virus at 2×10^{12} OB/L (300 OB/cell at 7×10^9 cell/L). However, economic modelling suggests yields need to be improved 4-fold further over this level to be commercially viable. While cell densities can potentially be increased 2-fold using improved fed batch processes, OB/cell yields need to be enhanced as well for economic success.

Lenz et al. (1991) has shown that it is feasible to achieve a 2-fold increase in OB/cell yields by cloning the same *Heliothis* cell line used in this study. However, these clones were selected on the basis of static culture screening processes using serum containing media, and there is no guarantee that cells in static culture will adapt and perform consistently in suspension culture. In this study clone selection on the basis of virus yield in suspension culture using an in house low cost serum free medium was carried out.

Clearly a clone that produces double current OB/cell yields in a stable manner would be an ideal outcome. However, success in generating a clone with a similar yield to that of the current mixed cell population would be beneficial for manufacturing purposes. A cloned cell line is more likely to provide consistent yields in a manufacturing process, than a mixed population, where faster growing cells and non-producers gradually become more abundant (Kromenaker and Srienc, 1994). In addition if modification genetically of the host cell is required to get the desired increase in yield, then modification genetically of a cloned cell line is potentially more likely to succeed than manipulation genetically of a mixed population.

2. Materials and methods

2.1. Insect cell lines, media and passaging

The *H. zea* cell line, designated as BCIRL-HZ-AM1, was isolated from pupal ovarian tissue by McIntosh and Ignoffo (1983). The cell line maintained in the serum supplemented medium TC 199-MK, was provided at passage 242 as a static flask culture. These cells were eventually adapted to a low cost, in-house, serum free media VPM3 (Virus Production Medium) medium, which was disclosed in the PCT patent, WO/2005/045014 (Lua and Reid, 2005). *H. zea* cells were typically cultivated in 250 ml Erlenmeyer flasks containing 50–100 ml of VPM3 medium at 28 °C, and grown on an orbital shaker operating at 120 rpm. Cells were routinely passaged twice a week, with a seeding cell density of $2–4 \times 10^5$ cells/ml. Viable cell counting using a 0.1% trypan blue exclusion method was used to quantify cell growth, and estimation of errors by this method was described by Nielsen et al. (1991).

2.2. Virus strain and infection

The *H. armigera* single-nucleocapsid nucleopolyhedrovirus, HaSNPV (Family *Baculoviridae*, Strain H25EA1 isolated by CSIRO Entomology, Canberra, Australia), was used for infections. This is an Australian wildtype field isolate.

The procedure for virus stock preparation, infection, serial passaging of budded virus (BV) and OB production followed strictly the lab scale protocol mirroring the industrial in vitro baculovirus production process described by Lua and Reid (2005). Briefly, 5×10^9 caterpillar derived OBs were digested with an alkaline solution for 30 min, neutralized by the addition of VPM3, filter sterilized and inoculated to a total culture volume of 100 ml containing *H. zea* cells at 5×10^5 cells/ml. 15% v/v of passage 1 (P1), BV harvested at 4 d.p.i. was used for P2 virus production. 15% v/v of P2 BV at 3 d.p.i. was used for P3 infections.

2.3. OB assay

For OB per cell estimation, total cell densities were manually counted every 24 h during the first 3 days of the P3 infection to estimate peak cell densities post infection. At 7 d.p.i. of P3 infection, 0.5 ml cell culture was sampled and digested in 0.5 ml SDS for 30 min before being diluted further in water for OB haemocytometer counting as described in Pedrini et al. (2005). OB per cell was determined by dividing the OB per ml count by the peak cell density.

SDS PAGE was carried out to confirm relative OB levels. 3×10^5 cells from 7 d.p.i. of P3 infected cultures were collected by centrifugation. Heat inactivation of proteases by incubating samples at 70 °C for 30 min was followed by OB solubilization using incubation at 28 °C in an alkaline solution (25 mM Na_2CO_3, 50 mM NaCl, PH = 11). 7×10^3 solubilized cells were loaded on to each well of NuPAGE® 4–12% Bis-Tris gels and run with MES buffer in an Xcell Surelock™ Mini-cell electrophoresis apparatus according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The gel was stained using Bio-safe Comassie Blue (Bio-rad, Hercules, CA), destained with deionized water and photographed.

2.4. BV assay

BV titre was determined by in silico prediction of the multiplicity of infection (MOI) of baculovirus-infected insect-cell suspension cultures via the Power-Nielsen model (Power et al., 1994). The original model (encoded in Matlab® programming language, Release R2009a, Mathworks, Natick, MA) was adapted to wild-type HaSNPV infections by Dr Leslie Chan, using virus kinetic data generated by Dr Marcia Pedrini (2003). Briefly, 50 ml shaker flask cultures (in 125 ml flasks) were seeded at 5×10^5 cells/ml and infected with 2.5% or 5% v/v of virus inocula. Cell densities were estimated at 0, 2, 3 and 4 d.p.i. to determine the cell density at the time of infection (TOI, 0 d.p.i.) and the peak total cell density (PTCD) for each shaker infection. The model was then run with the empirical TOI as a fixed input, and MOI as a variable input, until the simulated PTCD output