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New cell lines from Ephestia kuehniella: characterization and susceptibility to baculoviruses

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Abstract

New cell lines from embryos of *Ephestia kuehniella* were recently developed. Primary cultures were initiated in September 2002 from 2 to 4 day old eggs in either modified TC-100 or ExCell 400 medium. From these initial cultures, one, originally isolated in the Ex-Cell medium, produced sufficient cell growth to allow subcultivation and eventually led to the establishment of two cell strains, one that forms multicellular vesicles in suspension and one consisting of tightly attached epithelial-like cells. The strains were compared to an extract from *E. kuehniella* eggs by isozyme analysis and shown to be from the same species. Both strains were inoculated with various insect viruses, including nucleopolyhedroviruses from *Autographa californica, Anagrapha falcifera, Anticarsa gemmatalis, Galleria mellonella, Heliothis armigera, Helicoverpa zea, Lymantria dispar, Plutella xylostella,* and *Rachoplusia ou*. Both strains were highly susceptible to most of the nucleopolyhedroviruses (with the exception of the viruses from *Helicoverpa zea* and *Lymantria dispar* which did not show cytopathology to either cell strain) with large numbers of occlusion bodies produced in most of the inoculated cells. Our results suggest these new lines can be useful in biocontrol research.

Keywords: Ephestia kuehniella cell lines, Baculoviridae, Nucleopolyhedrovirus

Abbreviation:

NPV	nucleopolyhedrovirus, the prefix M or S refers to multiple or single nucleocapsids
Ek-x4T	an attached cell line from *E. kuehniella*
Ek-x4V	a vesicular cell line from *E. kuehniella*

Introduction

The Mediterranean flour moth, *Ephestia kuehniella* (Keller)(Lepidoptera: Pyralidae), is a serious pest of stored food products, especially whole and milled grains. Its close association with human foods makes it a prime target for control methods other than chemical pesticides. The insidious flower bug, *Orius insidiosus* (Say)(Hemiptera: Anthocoridae), is a common predator of insect pests and is used for biological control in commercial flower and vegetable greenhouses (Barber, 1936; Assoc. Nat. Biocont. Prod., www.anbp.org). The producers of beneficial insects generally use eggs of *E. kuehniella* that are expensive to rear for the predator (e.g., $1400/kg, Koppert Biological Systems, www.kopert.com). *O. insidiosus* can be reared on an artificial diet that is economical to produce (Weiru and Ren, 1989); however, the fecundity of the females is significantly less than those reared on *Plodia interpunctella* or *E. kuehniella* eggs (Shapiro and Ferkovich 2002; Ferkovich and Shapiro, in press). We have found that supplementing the artificial diet with cells from a continuous insect cell line developed from *P. interpunctella* embryos significantly improved the fecundity of the adults (Ferkovich and Shapiro, unpublished). However, we have also found that supplementing diet with extracts of *E. kuehniella* eggs improved adult fecundity to a greater degree than either the eggs or the cell line of *P. interpunctella*. Thus, we believed a cell line from *E. kuehniella* embryos might provide a better supplement for the *O. insidiosus* diet. Since no such cell line previously existed, we undertook efforts to develop one.

In this study, we used *E. kuehniella* embryos to initiate primary tissue cultures. From eight initial primary cultures, one had sufficient cell growth to subculture and led to the development of a continuous cell line, the first reported from this insect. Subsequent selection methods yielded two strains with different morphologies. The cells were characterized by morphology and isozyme analyses. They were subsequently screened for replication of various NPVs and shown to be susceptible to *Autographa californica* MNPV, *Anagrapha falcifera* MNPV, *Anticarsa gemmatalis* MNPV, *Galleria mellonella* MNPV, *Heliothis armigera* MNPV, *Plutella xylostella* MNPV, and *Rachoplusia ou* MNPV but

with no apparent susceptibility to *Helicoverpa zea* SNPV or *Lymantria dispar* MNPV. Thus, these lines may be useful in the study and/or production of some of these baculoviruses. Studies are currently underway to determine if the initial goal of improving diets for *O. insidiosus* using the *Ephestia* cell line has also been achieved.

Methods and Materials

Cell line development. Primary cultures were initiated from 2 to 4 day old *E. kuehniella* eggs using procedures described in Lynn (1996). Primary cultures were initiated in BML-TC/10 medium (=TC-100 from Sigma Chemical Co., , www.sigmaaldrich.com) modified as previously described (Lynn et al. 1988) or in Ex-Cell 400 medium (JRH Biosciences, www.jrhbio.com) supplemented with 10% fetal bovine serum (Intergen, www.intergenco.com) in September 2002 and maintained at 26° C. Medium was added or replaced on these cultures at 7-14 day intervals until enough cells were available for subculturing. Of the initial eight cultures, one initiated in Ex-Cell 400 medium (Ek-x4) had sufficient growth to form a cell line.

During early medium replacements, the cells in the Ex-Cell 400 medium culture showed heterogeneous morphologies between the attached and suspended cell types (Fig 1A and 1B). Two strains were isolated from this culture by selecting for attachment and non-attachment, respectively. Attached cells were obtained by replacing the medium with 2.0 ml trypsinization buffer (Ca- and Mg-free Hanks' phosphate buffered saline, Lynn, 1996). This rinse was discarded and an additional 1.0 ml of buffer containing 50 µg/ml VMF trypsin (Worthington Enzymes, Freehold, NJ) was added. After 10 minutes, fresh Ex-Cell 400 medium containing 10% fetal bovine serum was added and the contents distributed to new flask(s). The strain that arose from this procedure was designated IPLB-Ek-x4T (for trypsin).

Most of the viable cells in suspension were in aggregates in the form of fluid-filled vesicles (Fig. 1B). These cells were selected by gently removing medium from the primary culture, slightly disrupting the vesicles by drawing the medium into a pipette multiple times followed by a low speed (50 x g) centrifugation for 5 min. The resulting pellet was resuspended in fresh Ex-Cell 400 medium supplemented with 5% fetal bovine serum and transferred to a fresh tissue culture flask. This strain was designated IPLB-Ek-x4V (for vesicle).

Maintenance. The subcultivation procedures described above have been continued with the two strains to maintain their respective morphologies. After the cultures could be subcultured, the cells were maintained in Greiner® tissue culture flasks. In the early stages of establishment, all cultures were split 1:2 after they had reached sufficient density as judged by microscopic examination, often taking 2 to 4 weeks between subcultures. The split ratios were altered as the cell growth rate increased during adaptation to the culture conditions. Both strains are currently subcultured on a one-week schedule at a 1:3 split ratio. It was also determined after a few passages that cells in both strains grew better in Ex-Cell 400 medium supplemented with only 5% fetal bovine serum (rather than the 10% used in the primary cultures). Cells did not grow well if the serum was completely omitted.

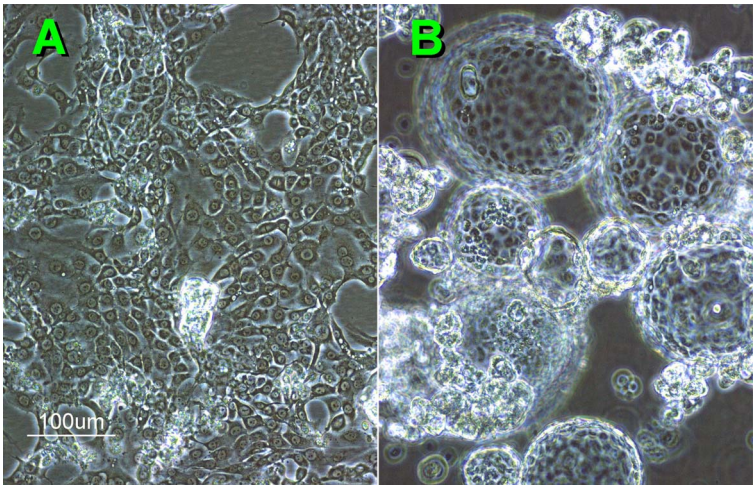

Figure 1 . Phase contrast micrographs of new *Ephestia kuehniella* cell lines at five days post subcultivation. 1A. IPLB-Ekx4T; 1B. IPLB-Ek-x4V. Both figures are at the same magnification. Marker bar is 100 µm.

Characterization. Extracts of the cells from both strains were prepared using the Authentikit® isozyme system (Innovative Chemistry, www.innovativechem.com)following the manufacturer's directions. The extracts were applied to 1% agarose gels, electrophoresed at 160 V for 25 min and then stained for malic enzyme (ME, E.C. 1.1.1.40), isocitrate dehydrogenase (ICD, E.C. 1.1.1.42), phosphoglucose isomerase (PGI, E.C. 5.3.1.9), or phosphoglucomutase (PGM, E.C. 2.7.5.1). The stains, buffers and gels were obtained from Innovative Chemistry (www.innovativechem.com). An extract from IPLB-Sf21cells (Vaughn et al., 1977) and another prepared from *E. kuehniella* eggs were also included on the gels for comparison and the isozyme migration patterns on these gels were also compared with gels of other cell lines maintained in our laboratory.

Virus studies. The viruses tested in these studies included the following clonal isolates: *A. californica* NPV-pxh, *A. falcifera* NPV-sf, *A. gemmatalis* NPV-ag3, *G. mellonella* NPV-r2/f6, *H. armigera* NPV-r2/b10, *H. zea* SNPV-hv1, *L. dispar* NPV-a624, *P. xylostella* NPV-ep/h10, and *R. ou* NPV-tnr. The virus inocula were all derived from cell cultures that are permissive for the respective viruses and were at passage level 2 or 3. The original source of most of these viruses has been previously described (Lynn and Shapiro, 1998; Lynn, 2003) although in some cases, the clones used in this study were recently selected (unpublished data).

To screen for virus susceptibility, cells of each strain were transferred to 24-well tissue culture plates (Greiner®) in 0.5 ml Ex-Cell 400 medium supplemented with 5% fetal bovine serum and 50 µg/ml gentamicin sulfate. Fifty µl of each virus inocula were added to each well. Due to the growth characteristics of some of the *E. kuehniella* cell strains, accurate cell counts were not possible; however, based on microscopic examination of the cultures, we estimated that the virus inocula used in these studies represent a multiplicity of infection of 0.1-1 infectious particles per cell. In our experience, this level of inoculation should be adequate for determining susceptibility of these cells to the various viruses. The inoculated plates were sealed with masking tape, maintained at 20°

C (shown in previous studies to improve observation of virus in insect cell cultures, Lynn, 2002) and examined/photographed with a Nikon TE300 inverted microscope equipped with both phase contrast and Hoffman modulation contrast optics at intervals up to 1 month for the presence of viral OBs.

Results and Discussion

Two continuous cell strains have been established from embryos of the Mediterranean flour moth. The morphologies of these cells are seen in Figure 1. Ek-x4T cells are predominantly epithelial-like, firmly attached cells although some cells are more fibroblastoid, especially at lower cell densities (Fig. 1A). Ek-4xV cells grow in large aggregates of suspended cells with many cells occurring in well-organized vesicles (Fig. 1B). This morphology is seen occasionally in insect cell cultures and is usually representative of epithelial cells (Lynn et al., 1982).

The similarity of the isozyme patterns of these cells to *E. kuehniella* egg extract (Fig. 2) indicates that they are derived from *E. kuehniella*. Although the extract from the eggs had lower enzymatic activity, the bands that could be seen in those lanes (marked as "eggs" in Fig 2) matched those present in both cell strains. While the migration patterns in the new cell strains do not differ from each other, they were different in each enzyme system except for the gel stained for PGI from that seen with IPLB-Sf21 cells. In addition, comparing patterns previously obtained with other continuous cell lines maintained in this laboratory (data not shown) showed that the Ek-x4 strains are unique, verifying they are new cell lines.

Both of the new lines were highly susceptible to five of the viruses originally isolated from noctuid insects (*A. californica, A. falcifera, H. armigera* and *R. ou*) as well as viruses from *G. mellonella* and *P. xylostella* while being only slightly susceptible to *A. gemmatalis* NPV, and with no apparent susceptibility to the NPVs from *H. zea* or *L. dispar* (Table 1 and Fig. 3). Since most of the viruses infectious to these cells are thought to be closely related it is not surprising that the cells are susceptible to them. However, given that the viruses are mostly from noctuid moths, while the

Table 1. Replication of nucleopolyhedroviruses in new cell lines from *Ephestia kuehniella*.

Virus	IPLB-Ek-x4T	IPLB-Ek-x4V
Autographa californica nucleopolyhedrovirus	+++	+++
Anagrapha falcifera nucleopolyhedrovirus	+++	+++
Anticarsa gemmatalis nucleopolyhedrovirus	+	+
Galleria mellonella nucleopolyhedrovirus	+++	+++
Heliothis armigera nucleopolyhedrovirus	+++	+++
Heliothis virescens nucleopolyhedrovirus	+++	+++
Helicoverpa zea nucleopolyhedrovirus	-	-
Lymantria dispar nucleopolyhedrovirus	-	-
Plutella xylostella nucleopolyhedrovirus	+++	+++
Rachoplusia ou nucleopolyhedrovirus	+++	+++

\+ Complete replication with the formation of occlusion bodies in <20% of cells by 1-week post infection.
+++ Complete replication with the formation of occlusion bodies in >80% of cells by 1-week post infection.
\- No apparent infection by visual inspection with phase contrast or Hoffman modulation contrast microscopy.

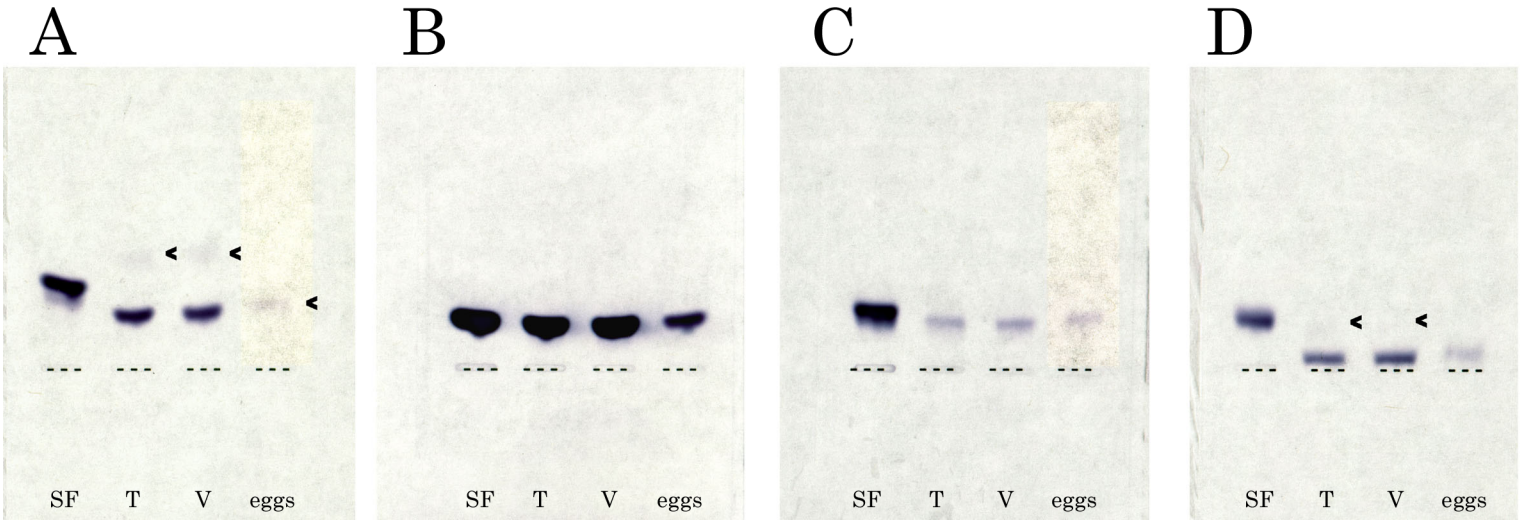

Figure 2. Electrophoresis patterns on gels stained for 2A. phosphoglucomutase, 2B phosphoglucose isomerase; 2C. isocitrate dehydrogenase; or 2D Malic enzyme . All materials were from the Authentikit® system. The — mark shows the location were the individual cell extracts (from the cell line indicated at the bottom of the figure) were applied to the gels and the arrow heads < indicate the location of some light bands that were seen on the gels but which may not be apparent in the photo. Samples were electrophoresed 25 min prior to staining for the indicated substrate.

cells are from a pyralid, it is somewhat surprising that they are susceptible at all. However, these particular virus isolates are known to have a broad host range, although they have never been shown to be infectious to E. kuehniella larvae.

These new lines appear to have good potential for studying baculoviruses and, with some further optimization of the growth conditions, they may also be useful in a production scheme for some of these viruses. We are also currently examining the potential of these cells to enhance the fecundity of *O. insidiosus* reared on artificial diets.

Acknowledgements

We thank Ms. Vallie Bray for preparation of media and supplies. Mention of proprietary or brand names is necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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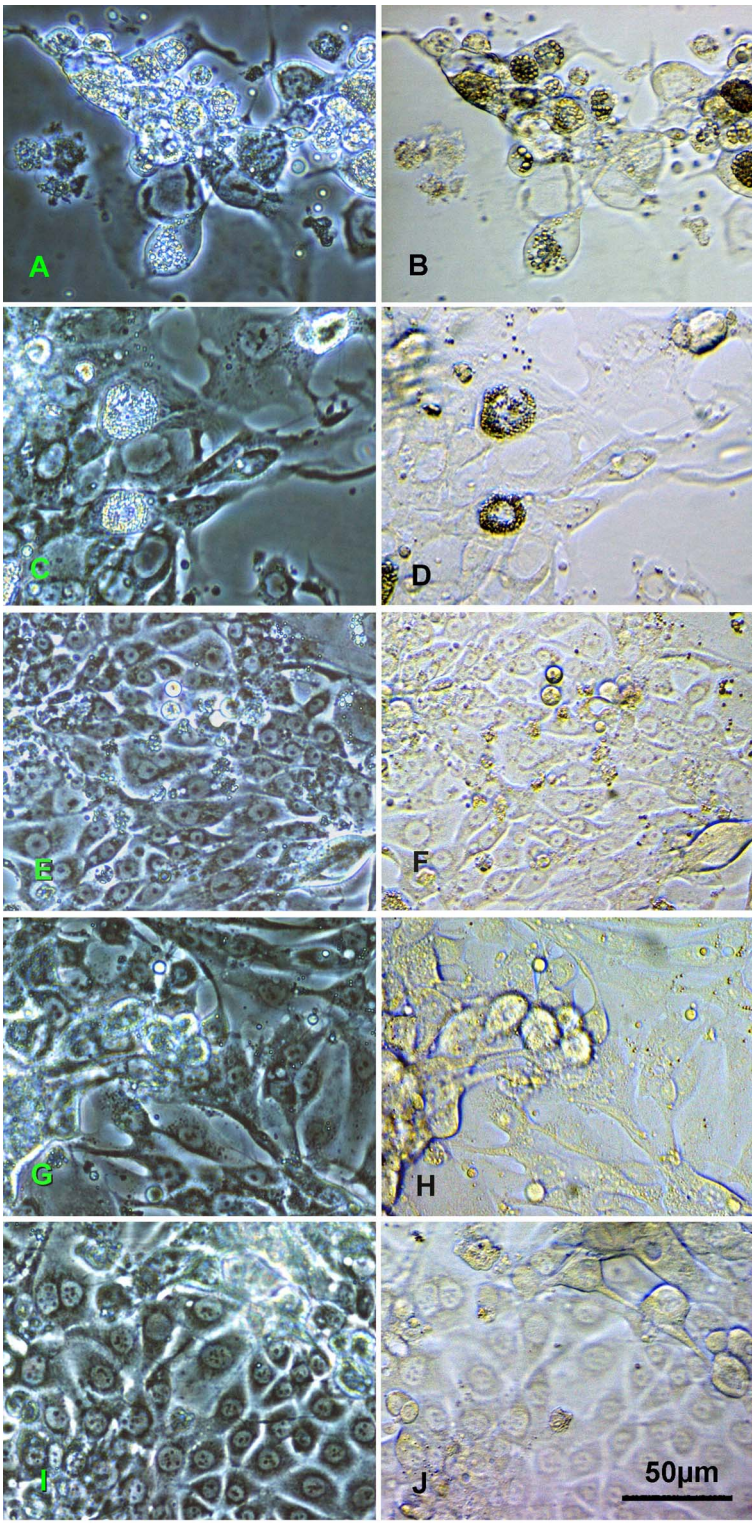

Figure 3. IPLB-Ek-x4T cells 6-days post inoculation with virus. 3A and 3B, *A. californica* NPV-infected cells; 3C and 3D, *A. gemmatalis* NPV-infected; 3E and 3F, *H. zea* SNPV-inoculated; 3G and 3H, *L. dispar* NPV-inoculated; and 3I and 3J, IPLB-Ek-x4V control cells at the same time. Figures 3A, 3C, 3E, 3G, 3I taken with phase contrast optics; 3B, 3D, 3F, 3H, and 3J all taken using Hoffman modulation contrast, which better reveals the occlusion bodies in the nuclei of infected cells. All micrographs are at the same magnification as indicated by the 50µm marker bar in figure 3J.

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