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REPORT

Establishment of two midgut cell lines from the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract

Two cell lines were generated from larval midguts of *Spodoptera frugiperda* and have been 26 passaged over 50 times. The CT/BCIRL-SfMG1-0611-KZ line was established from 27 trypsinized, minced whole midgut tissues: the CT/BCIRL-SfMG-0617-KZ line from isolated 28 midgut muscle tissue (containing some residual epithelial cells). Additional midgut cultures were 29 generated from isolated epithelial cells; some passaged not more than three times, which grew 30 very slowly and survived longer than 1 year. The continuously replicating cell lines contain 31 firmly adhering cells with different morphologies, including elongated, spherical, and/or 32 rectangular. The mean diameters of these cell lines are 9.3 ± 4.0 μm (SfMG1-0611) and 9.2 ± 3.9 33 μm (SfMG-0617). Growth curves for the two lines have relatively lengthy doubling times of 73.9 34 h and 50.4 h for SfMG1-0611 and SfMG-0617, respectively. We confirmed the identity of these 35 lines using DNA amplification fingerprinting (DAF-PCR) and noted that the DNA patterns for 36 each cell line were similar to their host tissues but distinctly different from other cell lines or 37 tissues from different insect species. Amplification of genomic DNA with species-specific 38 primers yielded DNA fragments of the expected sizes and with sequences nearly identical to 39 those from the *S. frugiperda* genome. Both cell lines were exposed to selected *Bt* Cry proteins 40 with minimal impact. These lines are currently available to researchers worldwide.

Keywords Hepatocytes · Liver sinusoidal cells · Bovine liver cell lines · Cytochrome P450 · mRNA expression

Cell lines have gradually come to play an important role in almost every aspect of insect science, including basic and applied studies. Next-generation cell lines refer to insect cell lines that are initiated from specialized tissues, midgut, for example, or from insect taxa that are not represented in current insect cell line collections, such as honeybees (Reall et al. 2019). Developing lines with in vivo cell properties and functionalities continues to be the most challenging aspect of next-generation cell line establishment, although there have been some successes. A cell line derived from *Spodoptera exigua* larval hemocytes retained its ability to phagocytose microspheres (Yasunaga-Aoki et al. 2004). Gao et al. (1999) exposed the mosquito cell line Aag-2 to bacteria and stimulated the production of antimicrobial proteins similar to those produced in vivo.

We report here on the establishment of two fall armyworm (*Spodoptera frugiperda*) cell lines from larval midguts. *S. frugiperda* is an agricultural pest of the Western Hemisphere and, most recently, the pest has become a new invasive species in West and Central Africa where the first outbreak was recorded in early 2016 (Goergen et al. 2016), as well as in India and throughout Asia (Reall et al. 2019, https://www.cabi.org/isc/datasheet/29810 [accessed 8/29/2019]). Fall armyworm cell lines are commonly used for the production of recombinant proteins, often in combination with baculovirus expression vectors (Elias et al. 2007). Examples are the Sf21 cell line and its clonal line Sf9, originally established from ovarian tissue (Vaughn et al. 1977). A midgut cell line from this economically important species is needed for research and high-throughput screening purposes.

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Cell lines were initiated from fifth or sixth instar larvae obtained from Benzon Research Inc., Carlisle, PA, and reared on diets provided with the larvae at 28°C, 60–70% humidity. Larvae were surfaced sterilized in 3–5 mL 0.825% sodium hypochlorite (3 min) and 70% ethanol (3 min), and then briefly rinsed in calcium, magnesium free phosphate buffered saline (CMF-PBS) containing antibiotics (200 U/ml penicillin, 0.2 mg/ml streptomycin). The peritrophic membrane, containing the food bolus, was removed. Midguts were washed three times in Hanks Balanced Salt Solution (ThermoFisher Scientific, Waltham, MA) containing the above antibiotics. In some cases, tissues were incubated with 0.5% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) for 4 min while mincing, then medium containing 9% heat-inactivated FBS (# F6178, Sigma-Aldrich), with the antibiotics just mentioned, was added and the suspension was centrifuged (800×*g*, 10 min, 4°C). Medium (2.5 to 5 mL) with 9% FBS and antibiotics was added to the resulting pellet and the suspension was transferred into either a standard tissue culture $T_{12.5}$ or T_{25} flask or a poly-D-lysine-treated/collagen-treated T_{25} flask (Corning Inc., Durham, NC). To isolate midgut epithelial cells, the procedure of Hakim et al. (2009) was followed. Briefly, midgut tissues were shaken on an orbital shaker (1.5 to 2 h, 60 rpm), pipetted up and down to enhance cell dissociation, and strained through a 70 μm nylon sieve (Fisher Scientific, Waltham, MA). The tissue filtrate was centrifuged (800×*g*, 10 min, 4°C) and the resulting pellet was re-suspended in 3–5 mL medium containing 9% FBS with the above antibiotics and transferred into $T_{12.5}$ or T_{25} flasks.

Eleven cell culture media were tested: EX-CELL 405, 420 and TiterHigh (Sigma-Aldrich), TNM-FH, IPL-41, L15B, Shields and Sang, RPMI 1640, DMEM/Ham's F-12 (Caisson Laboratories, Smithfield, UT), Kimura's (Kimura 1984), and Sf900-II (Fisher Scientific). Cultures were maintained at 28°C, monitored weekly, and fed by replacing half the spent medium with fresh medium (containing reduced levels of antibiotics: 50 U/ml penicillin, 0.05 mg/ml streptomycin) as needed until confluency. For passaging, 0.5% trypsin-EDTA (Sigma-Aldrich) or TPP cell scrapers (MIDSCI, St Louis, MO) were used to remove the firmly attached cells from the flask bottoms.

Ninety-seven midgut cell cultures were initiated from 5th/6th instar *S. frugiperda* larvae using a variety of media and conditions. The only medium that supported continuous proliferation of the midgut cells was TNM-FH + 9% FBS. The two midgut cell lines that have been continuously replicating for over 50 passages were generated from trypsinized, minced whole midgut tissues from 6th instars placed into a poly-D-lysine-treated flask (CT/BCIRL-SfMG1-0611-KZ); and 5th instar midgut tissue recovered from the 70-μm filter that was transferred into a standard tissue culture flask (CT/BCIRL-SfMG-0617-KZ). We use the abbreviation SfMG to refer to both cell lines in the text. These cell lines contain firmly adhering cells that are detached using a cell scraper, because 0.5% trypsin treatments led to limited cell detachment. The SfMG1-0611 cells have a mean cell diameter of 9.3 ± 4.0 μm with different morphologies, including elongated, spherical, and rectangular (Fig. 1*A*, *C*). The SfMG-0617 cells have a mean diameter of 9.2 ± 3.9 μm and have a more uniform spherical or rectangular shape (Fig. 1*B*, *D*). Both cell lines are mycoplasma-free based on the MycoAlert™ Kit (Lonza, Allendale, NJ).

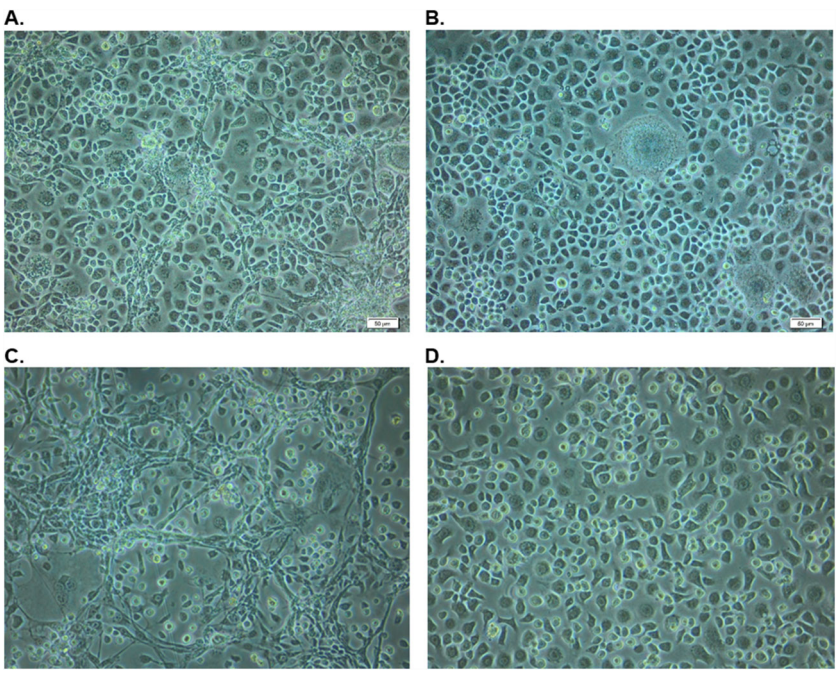

Figure 1. Two midgut cell lines from *S. frugiperda*. (*A*) SfMG1-0611, passage 4, 200×. (*B*) SfMG-0617, passage 5, 200×. (*C*) SfMG10611, passage 32, 200×. (*D*) SfMG-0617, passage 33, 200×.

Growth curves were generated for each Sf midgut cell line by seeding the cells into $T_{12.5}$ flasks (2×10^5 cells/ml, 2.5 mL/flask) and monitoring concentrations of 0.4% trypan blue-stained cells every 2 d (3 flasks per time point) using a Cellometer® (Nexcelom Bioscience, Lawrence, MA). The linear portion of the growth curves were used to determine the doubling times (Roth V. 2006 Doubling Time Computing, http://www.doubling-time.com/compute.php): 73.9 h (SfMG1-0611) and 50.4 h (SfMG-0617).

The identities of the midgut cell lines were authenticated using two PCR-based techniques. For both procedures, genomic DNA was extracted from the cell lines and tissues using the DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany). In the first analysis, we used species-specific primers: Sf COI-F TTT GGA ATT TGA GCA GGG ATA GTA GG ACC, Sf COI-R ACC ATG AGC AAT ATT AGA GGA GA (cytochrome c oxidase subunit I, KM362176.1); Sf HSP70-F TCG ATG CCA AGC GTC TTA TT, Sf HSP70-R CTG ATG TCC TTG CCC TTC TT (heat shock protein 70, AY161271.1); and Sf HSP90-F GGA GGA CAA GAA GGA CAA GAA G, Sf HSP90-R GAC TCG CTC TAC GAA TGA TGA G (heat shock protein 90, AF254880.1). A Veriti thermal cycler (Applied Biosystems, Waltham, MA) was used with the following program for amplification: 95°C for 5 min; 30 cycles of 95°C for 60 s, 55°C for 60 s, and 72°C for 120 s; and a final 72°C for 5 min. The PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN) and then sequenced by the University of Missouri DNA Core Facility (Columbia, MO). The resulting sequences were used to BLAST search the NCBI database to confirm the identity of the cell lines. PCR of genomic DNA from either whole larvae or the SfMG cell lines using these primer sets resulted in amplification of a single DNA product with 98–99% identity for all PCR products with published *S. frugiperda* sequences for these genes (Table 1). The second analysis involved gene primers used for DNA amplification fingerprinting (DAF-PCR) as described by McIntosh et al. (1996). DAF-PCR analysis yielded cell line DNA patterns (SfMG1-0611 and SfMG-0617) similar to their host, Sf tissues, but distinctly different from other cell lines or tissues from either different species within the same genus or other insect orders (Coleoptera, Diptera, Hemiptera) (Fig. 2).

Cytotoxicity assays were performed using the SfMG1-0611, SfMG-0617, and Sf9 (positive control). Cells were seeded at 4×10^5 cells/ml into a 96-well plate (50 µL/well). Serial concentrations (1:10) of activated *Bacillus thuringiensis* (*Bt*) Cry1Ca or Cry1Fa proteins (Abdelgaffar et al. 2019), or the Cry3Aa protoxin (negative control), were added to the wells (0.002 to 200 µM; 50 µL/well) and the plates were incubated overnight (28°C). CellTiter Blue dye (20 µL/well; Promega, Madison, WI) was then added to the wells (2 h, 28°C) and the resulting fluorescence was measured (560/590 nm). Data were analyzed by ANOVA with Fisher's LSD multiple-comparison test using NCSS 10 Statistical Software (2015, NCSS, LLC. Kaysville, Utah, ncss.com/software/ncss). Reciprocal transformation was used for the SfMG-0617 data, as values were otherwise not normally distributed. Neither the viability nor cell number of the *S. frugiperda* midgut cell lines were impacted by the *Bt* proteins Cry1Ca (Fig. 3) or Cry1Fa (data not shown), although the positive control (Sf9) exhibited cell lysis and decreased cell numbers in a dose-dependent manner for Cry1Ca as expected (Fig. 3). These data suggest that the SfMG cells did not express Cry protein receptors.

Many of the established fall armyworm cell lines now available were derived from imaginal discs, testes, or ovarian tissues, such as Sf21 that was established from ovarian tissues (Vaughn et al. 1977). Our cell lines were initiated from midgut tissues, the main entry for pathogens and pest control agents. Insect midgut epithelial tissues largely consist of stem cells, columnar cells, and goblet cells (Caccia et al. 2019). The stem cells are the adult midgut precursor cells that remain undifferentiated. Columnar cells (a.k.a. enterocytes) and goblet cells are differentiated from stem cells and then enlarge the midgut

Table 1. Characterization of SfMG cell lines using PCR. PCR amplicons were purified, sequenced, and the resulting sequences were used to BLAST search the NCBI database to confirm the identity of the cell lines

Gene	Values	SfMG1-0611	SfMG-0617
Cytochrome oxidase subunit I	Score (bits)	558	551
	Expect	2.00E−156	2.00E−156
	Identity (%)	310/314 (99%)	310/314 (99%)
Heat shock protein 70	Score (bits)	887	1323
	Expect	0	0
	Identity (%)	502/512 (98%)	744/758 (98%)
Heat shock protein 90	Score (bits)	1195	1181
	Expect	0	0
	Identity (%)	663/671 (99%)	661/672 (98%)

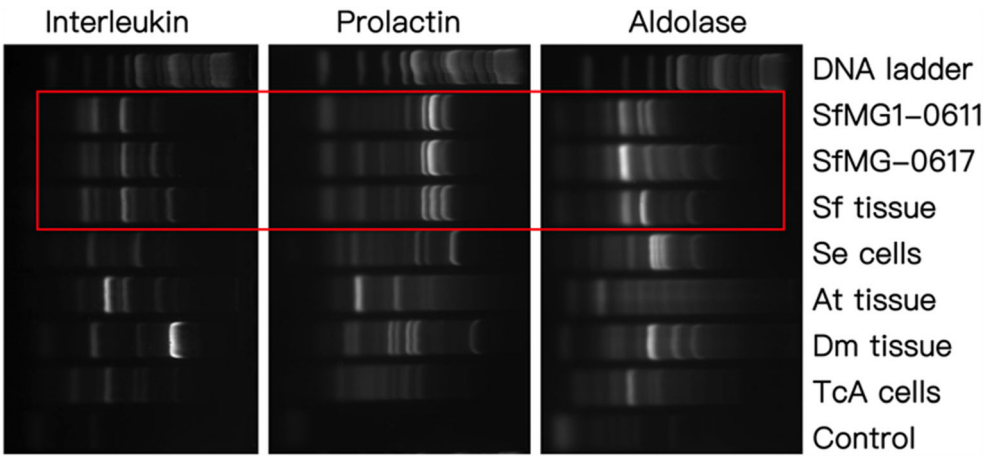

Figure 2. DAF-PCR authentication of the *S. frugiperda* midgut cell lines (SfMG1-0611, SfMG-0617) using 3 primers (interleukin, prolactin, and aldolase). Sf tissue = larval *S. frugiperda* tissue; Se cells = AMCY/BCIRL-SeE-CLG1, *Spodoptera exigua* embryonic cell line; At tissue = *Anasa tristis* tissue; Dm tissue = *Drosophila melanogaster* tissue; TcA cells = BCIRL-TcA-CLG1, *Tribolium castaneum* pupal/adult cell line.

during molts. The midgut cell lines reported here do not have the morphologies typically associated with midgut epithelial cells. Primary cultures initiated from isolated stem cells having these morphologies did not remain viable beyond three passages. Of the two cell lines that were generated, the SfMG1-0611 line originated from whole midgut tissues, which initially consisted of both epithelial and muscle cells. SfMG-0617 was established from the tissue remaining after filtration, which consisted primarily of muscle tissue but also included some epithelial cells (Hakim et al. 2009).

Midgut cell lines have been generated from a number of lepidopteran species. These lines exhibit many properties, including a variety of doubling times and different degrees of responsiveness to *B. thuringiensis* Cry proteins or baculoviruses. Midgut cell lines from *Choristoneura fumiferana*, *Bombyx mori*, and *Lymantria dispar* have doubling times exceeding 1 wk (Baines et al. 1994), whereas the doubling time for a *Trichoplusia ni* cell line was 25 h (Granados, 1994) and that of an *Helicoverpa armigera* midgut cell line was 58.6 h (Li et al. 2015). The mean diameter of the *H. armigera* midgut cells (13.8 ± 1.8 μm) is slightly larger than the *S. frugiperda* midgut lines reported here. The *H. armigera* line is permissive to infection by two baculoviruses (*Autographa californica* nuclear polyhedrosis virus, AcMNPV, and *H. armigera* nuclear polyhedrosis virus). Two midgut cell lines have been established from *Helicoverpa zea* (Pringle et al. 2003; Goodman et al. 2004), with one responsive to Cry proteins (RP-HzGUT-AW1; Wei et al. 2018). The *C. fumiferana* midgut line, FPMI-CF203, was responsive to Cry proteins and the spruce budworm nuclear polyhedrosis virus (Sohi et al. 1993). A long-lived (but not immortal) midgut culture from *Pseudaletia unipuncta* survived for 24 mo with 6 passages, secreted peritrophic membrane-like secretions, and exhibited columnar and goblet cell morphologies. It was sensitive to a baculovirus (AcMNPV) (Garcia et al. 2001).

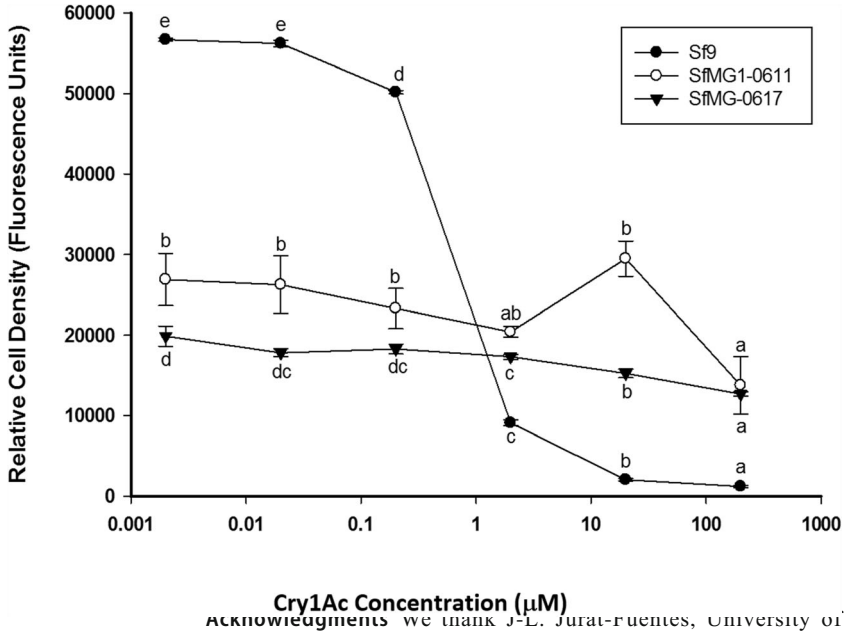

Figure 3. Impact of Cry1Ca on cell density of the Sf midgut cell lines (SfMG1-0611, SfMG-0617) and Sf9 (positive control). The Sf9 cell density declined in a dose-dependent manner, accompanied by cell lysis. Although densities of the Sf midgut cell lines declined in a statistically significant way, the declines were minor with no cell lysis. Values represent means ± SE (n = 3). Values for each cell line with different *letters* were significantly different.

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