This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier’s archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright
Development of primary cell cultures from the adult xylem-feeding leafhopper, Kolla paulula, as a tool for studying Wolbachia biology

Rong-Jen Shiau a, Hsien-Tzung Shih b, Shin-Yi Chen c, Chiou-Chu Su d, Wei-Huang Tsai e, Yu-Der Wen c,⁎

a Department of Beauty Science, Chienkuo Technology University, Changhua 500, Taiwan, ROC
b Applied Zoology Division, Taiwan Agricultural Research Institute, Council of Agriculture, Taichung 413, Taiwan, ROC
c Department of Biology, National Changhua University of Education, Changhua 500, Taiwan, ROC
d Department of Pesticide Application, Agricultural and Toxic Substances Research Institute, Taichung 413, Taiwan, ROC
e Plant Protection Division, Bureau of Animal and Plant Health Inspection and Quarantine, Taichung 100, Taiwan, ROC

ARTICLE INFO
Article history:
Received 15 April 2011
Revised 26 July 2011
Accepted 28 July 2011
Available online xxxx

Keywords:
Kolla paulula
Leafflower
Primary culture
Wolbachia

ABSTRACT

Xylophagous leafhoppers are vectors of xylem-limited plant pathogens such as citrus variegated chlorosis (CVC) and Pierce’s disease, which cause Xylella diseases. Currently, no cure for Xylella diseases exists. The objective of endosymbiont control using Wolbachia pipientis is to reduce the populations of insect vectors, potentially preventing the expansion of Xylella diseases. The purpose of this study was to establish primary cell cultures from adult xylem-feeding leafhoppers to study Wolbachia biology. Cells from adult male and female K. paulula (Walker) (Hemiptera: Membracoidea: Cicadellinae) were successfully cultured in Dulbecco’s Modified Eagle Medium containing 20% fetal bovine serum and were maintained for more than 6 months. Cells of both male and female adults are round and semi-attached. The doubling times for male and female K. paulula cells are approximately 8 and 10 days, respectively. The presence of Wolbachia in K. paulula cell cultures was detected by polymerase chain reaction (PCR) amplification of Wolbachia surface protein (wsp) gene. The cell cultures developed in this study may be useful in studying interactions between Wolbachia and its hosts of different genders.

© Korean Society of Applied Entomology, Taiwan Entomological Society and Malaysian Plant Protection Society, 2011. Published by Elsevier B.V. All rights reserved.

Introduction

Leaffhoppers are sap sucking insects that feed on leaf tissue, phloem sap, or xylem sap. This feeding behavior causes leaves to curl and is responsible for the transmission of numerous harmful pathogens (Ng and Falk, 2006; Purcell, 1982; Weintraub and Beanland, 2006). Xylophagous leafhoppers, for example, are vectors of Xylella fastidiosa, a xylem-limited gamma-proteobacterium. X. fastidiosa causes serious plant diseases such as citrus variegated chlorosis (CVC) and Pierce’s disease in grapevines (Redak et al., 2004). These diseases have caused considerable agricultural losses in the United States and in some South American countries (Curley et al., 2007; Redak et al., 2004). Currently, no effective treatment for Xylella diseases exists. To prevent the expansion of Xylella diseases, new strategies for pest management must be developed. Previous studies have proposed that symbiotic control using Wolbachia pipientis is a possible strategy to reduce the size of insect vector populations (Dobson, 2003; Marshall et al., 2011; Cook et al., 2008). W. pipientis is a maternally transmitted alpha-proteobacterium, which inhabits a variety of filarial nematodes and insect species (Werren, 1997). Based on the sequence data of Wolbachia surface protein (wsp), Wolbachia strains can be grouped into six supergroups (A–F). Strains belonging to supergroups A and B are frequently found in insect species (Werren et al., 1995; Zhou et al., 1998). Wolbachia infection results in reproductive abnormalities, including cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing (Stouthamer et al., 1999). Among Wolbachia-mediated reproductive defects, CI has been successfully applied to reduce the populations of medflies, mosquitoes, and planthoppers in laboratory studies (Noda et al., 2001; Xi et al., 2005; Zabalou et al., 2004).

Insect cell lines perform a vital function in Wolbachia applications. To apply the Wolbachia-mediated endosymbiotic control successfully, Wolbachia strains must adapt and proliferate in cells within target insects. Cell lines cultured in vitro, therefore, can serve as temporary hosts for the maintenance and amplification of Wolbachia strains (Dobson et al., 2002; Kawai et al., 2009; McMeniman et al., 2008; O’Neill et al., 1997). Insect cell lines are also used to study Wolbachia–host interactions. For example, by comparing proteomics from Wolbachia infected cell lines and from Wolbachia-free cell lines, researchers identified several host genes that might be involved in the processes of Wolbachia infection (Dangi et al., 2009; Fallon and Witthuhn, 2009; Hughes et al., 2011). Currently, insect cell lines are

⁎ Corresponding author.
E-mail address: ydwen@cc.ncue.edu.tw (Y.-D. Wen).

1226-8615/$ – see front matter © Korean Society of Applied Entomology, Taiwan Entomological Society and Malaysian Plant Protection Society, 2011. Published by Elsevier B.V. All rights reserved.
frequently used to study Wolbachia biology (Brennan et al., 2008; Frentiu et al., 2010; Kambris et al., 2010).

The present study was conducted to culture cells from three leafhoppers, *Kolla paulula* (Walker, 1858) (Hemiptera: Cicadellinae), *Idioscopus niveosparsus* (Walker, 1870) (Cicadellidae: Idiocerinae), and *Empoasca canara sonani* (Matsumura, 1931) (Cicadellidae: Typhlocybinae). *K. paulula* is a widespread xylem-feeding leafhopper in Taiwan (Shih et al., 2009) and is the most common xylem feeder in orchards in central Taiwan (Shih et al., unpublished data). Therefore, we hypothesize that *K. paulula* is a vector of *Xylella* diseases. *I. niveosparsus* is a phloem sap feeder that is a serious pest of mango (Backus et al., 2005; Chou and Chou, 1990). *E. sonani* feeds on leaf tissue (Gitau et al., 2009). Because these three species use different feeding behaviors and because all of these leafhoppers can be reared in the laboratory, we decided to develop cell cultures from these three leafhoppers as tools for further studies on the relationship between Wolbachia and its leafhopper hosts. Although cell lines are easier to establish from embryos and larvae, such cell lines have both male and female genetic backgrounds. In this study, we established cell lines from male or female adults to obtain unique sex backgrounds. Such cell lines derived from adults might be useful for studying the regulation of Wolbachia sex-specific expression genes such as *pk2* and the phage-related DNA methylase (Duron et al., 2007; Walker et al., 2007; Yamada et al., 2011).

Materials and methods

**Primary cell culture**

*K. paulula* adults were captured from fields in Douliou City, Taiwan, and *I. niveosparsus* and *E. sonani* adults were captured from fields in Wufeng City, Taiwan. All leafhoppers were reared in laboratory cages. Ten male or female adults were surface sterilized using a 75% ethanol solution for 10 min, followed by a 3% bleach solution for 5 min. The sterilization procedure was repeated three times. The insects were then washed three times in sterile phosphate-buffered saline (PBS). The heads and wings were removed and the rest of bodies were homogenized in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS). Tissue debris was removed by centrifugation and the supernatant was transferred to a tissue culture flask (Nunc, 25 cm²) containing DMEM and 20% FBS. The flasks were maintained at 28 °C in a humidified atmosphere.

![Fig. 1. Morphology of cells established from female *K. paulula* adults. Light microscopy on day 21 of culture.](image1)

![Fig. 2. Primary cultures from adult leafhoppers. Cells isolated from adult leafhoppers were cultured in DMEM supplemented with 20% FBS. Cell growth was monitored using light microscopy observation every week. The pictures of cells were taken with a digital camera in Weeks 1, 3, 8, and 9.](image2)
incubator. The culture medium was changed once per week. Cell morphology was observed by light microscope and pictures were taken using a digital camera. To measure the growth rates of primary cell lines, the cells (1.5 × 10⁶ cells/ml) were seeded in a tissue culture flask (Nunc, 25 cm²) containing 10 ml DMEM supplemented with 20% FBS. Ten microliters of cells were taken from the flask, stained with trypan blue, and counted using a hemocytometer under a light microscope every week.

**PCR-based detection of wsp gene**

Total DNA was extracted from adults or cell cultures using GeneSpin™-V2 Genomic DNA Isolation Kit (Bio-Protech, Taiwan) according to the manufacturer protocol. PCR was performed using total DNA as the template. The presence of Wolbachia was detected by PCR amplification using a fragment of wsp gene. The forward primer B81F (5'-TGGTCCAATAAGTGATGAAGAAAC) and reverse primers A691R (5'-AAAAATTAACGCCTAATCCA) or B522R (5'-ACCAGCTTGTGCTTATA) were designed according to a previous study (Braig et al., 1998). The PCR conditions were as follows: 95 °C for 1 min; 95 °C for 30 s, 55 °C for 1 min, 72 °C for 3 min (35 cycles); and 72 °C for 10 min. The amplicons were ligated into pGEM-T Easy cloning vectors (Promega) according to the instructions of the manufacturer. Clones containing the inserts were isolated and sequenced in an ABI 3730XL DNA Analyzer (Applied Biosystems; USA). The sequences were subjected to the GenBank for a homology search using the BLAST program.

**Phylogenetic analysis**

Multiple sequence alignment was conducted using the ClustalW program in BioEdit (Thompson et al., 1994). Phylogenetic analysis of wsp was carried out using the neighbor-joining method (1000 bootstrap replicates) in the MEGA 4.1 software (Tamura et al., 2007). The wsp gene of Coptotermes acinaciformis (Isoptera: Rhinotermitidae) (wCaci; accession number: AJ833931) was used as the outgroup.

**Results and discussion**

**Cell morphology and growth curve**

Morphology of adult male and female K. paulula cells was observed under a light microscope. The shape of both male and female cells was round. Cells were 3 to 7 μm in diameter and all cells were semi-attached (Fig. 1).

Fig. 2 shows that both male and female adult K. paulula cells grow rapidly in DMEM supplemented with 20% FBS. To test whether this medium can also support the growth of cells from other leafhoppers, we prepared cell cultures from I. niveosparsus, a phloem sap feeder, and E. sonani, a leaf tissue feeder. We found that cells derived from I. niveosparsus started to die in Week 3. By the end of Week 9, only a few cells were living in the medium. In contrast, E. sonani cells increased rapidly at the beginning. However, by the end of Week 9, the cell numbers had decreased significantly.

The growth curves of cells derived from male and female K. paulula adults were similar. K. paulula adult females had a faster growth rate. Both male and female cells reached their highest densities in Week 4 (5.6 × 10⁶ cells/ml for male cells and 6.12 × 10⁶ cells/ml for female cells) after which cell densities decreased. The doubling times for male and female cells were approximately 8 and 10 days, respectively (Fig. 3).

Several reports describe the preparation of cell lines from leafhoppers. For example, Kimura (1984) established primary cells from embryos of Nephotettix cincticeps, a vector that transmits the rice dwarf virus. The cells were maintained in Liu and Black’s medium. Kamita et al. (2005) established cell lines from Homalodisma coagulate (Germar, 1821), the glassy-winged sharpshooter (GWSS), a vector of X. fastidiosa. H. coagulate cells were cultured in Ex-Cell 401 medium containing 10% FBS or in LH medium containing 20% FBS. (H. coagulate was renamed Homalodisma vitripennis in 2006 (Takuya et al., 2006)). We established cell lines from K. paulula adults using DMEM supplemented with 20% FBS. This is the first report concerning primary cultures of K. paulula adults. Currently, we do not know why primary cell cultures could be established from K. paulula adults, but not from I. niveosparsus or E. sonani adults. One possibility might be due to the different food sources. I. niveosparsus feeds on phloem sap and E. sonani feeds on leaf tissue. This study sought to collect other
xylem feeding leafhoppers and test whether their cells can survive and proliferate in DMEM supplemented with 20% FBS.

Detection and identification of Wolbachia strains associated with K. paulula

To determine whether K. paulula is infected by single or multiple Wolbachia strains, two sets of primer pairs (B81F/A691R and B81F/B522R) were used in the reactions. As shown in Fig. 4, fragments of wsp can be amplified using both sets of primer pairs. The observed sizes of PCR products were similar to the predicted sizes, which were 610 bp and 440 bp, respectively. Results from the BlastN search showed that all PCR products were closely related to wsp, except the non-specific amplicon (Fig. 4B). The fragments of wsp amplified using B81F and A691R primers were similar to wAlbA (97% identities). The amplicons that were amplified using B81F and B522R primers were similar to wCon (98% identities) (data not shown). These two Wolbachia strains associated with K. paulula were designated as wKpauA and wKpauB. Previous studies have shown that wCon can cause CI in its insect host (Kondo et al., 2002). Therefore, it is necessary to test whether wKpauB can also induce CI in K. paulula. Moreover, the phylogenetic analysis based on the sequences of wsp showed that wKpauB was clustered with the Wolbachia strain associated with GWSS, but not with wZpul, the strain associated with Zygimida pulula (Bohemian; Hemiptera, Cicadellidae) (Fig. 5). Taken together, these results clearly demonstrated that K. paulula harbors at least two Wolbachia strains, and that these Wolbachia strains could be maintained in K. paulula cells cultured in vitro for a long period of time.

In conclusion, we successfully used commercially available DMEM supplemented with 20% FBS to culture primary cells from male and female K. paulula adults. It may be worthwhile to test whether this medium can be used to culture primary cells from other adult xylem feeding leafhoppers.

Acknowledgments

This work was funded by the Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, ROC grant 99AS-9.3.1-BQ-B2 and National Science Council, ROC grant NSC 98-2313-B-055-006-MY3.

References
