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Insect Biochemistry and Molecular Biology 36 (2006) 80–85
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

WO bacteriophage transcription in *Wolbachia*-infected *Culex pipiens*

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Received 12 July 2005; received in revised form 4 November 2005; accepted 4 November 2005

Abstract

Bacteriophages are commonly found in association with free-living bacteria, both as exogenic phages (virions) and as prophages integrated into the bacterial genome. In contrast, the observation of bacteriophages associated with obligate intracellular bacteria has been described infrequently. An exception is provided by *Wolbachia* endosymbionts, which harbor multiple phage elements that have been designated as WO phage. *Wolbachia* are maternally inherited bacteria that occur in the cytoplasm of many invertebrates, where they often manipulate host reproduction. Previously, the WO phage orf7 locus and ankyrin repeat-encoding genes have been observed to represent sources of genetic diversity between *Wolbachia* (*w*Pip) strains infecting mosquitoes of the *Culex pipiens* complex and have been suggested as potential participants in the reproductive manipulations. We have characterized WO phage associated with multiple *Wolbachia*-infected *Culex* strains and an uninfected strain using electron microscopy and RT-PCR. For each strain, different developmental stages were examined for transcription of three WO phage orf7 genes. The results provide evidence for the presence of both actively transcribed virions and inactive prophages. Variable orf7 transcription patterns are observed in comparisons of differing *Cx. pipiens* strains. Variability includes both mosquito stage-specific and sexually dimorphic orf7 expression patterns. This report provides additional support for the hypothesis that bacteriophages play an important role in *Wolbachia* and host evolution.

Keywords: *Wolbachia*; Phage WO; Gene expression; RT-PCR; Electron microscopy

1. Introduction

The importance of bacteriophages in the evolution of bacteria is increasingly apparent as additional bacterial genomes become available. Bacteriophages form up to 16% of bacterial genomes (Perna et al., 2001), and can affect the ecological, physiological and pathogenic character of bacterial species (Canchaya et al., 2003a, b; Ochman et al., 2000; Weinbauer and Rassoulzadegan, 2004). In contrast, intracellular bacteria are considered relatively devoid of foreign DNA in comparison with free-living bacteria. Obligate intracellular bacteria tend toward reduced genomes due to a lack of DNA repair mechanisms and a restricted environment, which acts to shelter their genome from the influence of foreign DNA (Bordenstein and Wernegreen, 2004; Tamas et al., 2002). Recently, two intracellular bacteria have been found to harbor bacteriophage elements: a gamma proteobacteria, secondary aphid endosymbiont (Sandstrom et al., 2001; Van Der Wilk et al., 1999) and intracellular *Wolbachia* endosymbionts (Fujii et al., 2004; Masui et al., 2000; Sanogo and Dobson, 2004; Wu et al., 2004).

Wolbachia endosymbionts have been documented across a wide range of invertebrate taxa, including insects, mites, isopods and filarial nematodes (reviewed in Stouthamer et al., 1999). *Wolbachia* induce a variety of phenotypic effects such as male killing, parthenogenesis induction, feminization, and cytoplasmic incompatibility. Cytoplasmic incompatibility (CI) is a common manifestation of *Wolbachia* in their hosts, occurring in matings between individuals infected with different *Wolbachia* types (Laven, 1967; O'Neill and Karr, 1990).

Wolbachia is an unusual obligate intracellular bacterium in that the chromosome of *Wolbachia* from *Drosophila melanogaster* (*w*Mel) contains up to 14% repetitive and

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doi:10.1016/j.ibmb.2005.11.001

mobile genetic elements (Wu et al., 2004). Bacteriophages and other mobile elements are hypothesized to have played an important role in the evolution of the *Wolbachia* genome due to duplication and rearrangement events (Bordenstein and Wernegreen, 2004; Wu et al., 2004). Prior experiments demonstrate that the WO bacteriophage is frequently associated with *Wolbachia* infections and include examples of exogenic, filterable bacteriophages and prophages integrated into the *Wolbachia* chromosome (Bordenstein and Wernegreen, 2004; Fujii et al., 2004; Gavotte et al., 2004; Masui et al., 2001; Sanogo and Dobson, 2004; Sinkins et al., 2005). The orf7 locus was one of the first WO sequences to be identified and is thought to encode a capsid protein (Masui et al., 2000).

In *Wolbachia*-infected *Culex pipiens*, the orf7 locus has been shown to represent a source of genetic divergence, allowing discrimination between *Wolbachia* infections occurring within different *Culex* strains (Sanogo and Dobson, 2004). Prior observation of phage-like particles in *Wolbachia*-infected *Culex* ovaries suggests the presence of an active WO phage (Wright et al., 1978). Furthermore, *Culex* strains differ in the number and type of orf7 loci (Sanogo and Dobson, 2004), suggesting that *Culex* individuals may be co-infected with multiple WO phage types.

To provide additional evidence that the identified orf7 loci in *Culex* represent active phage, primers specific for three orf7 loci have been used to examine transcription. An uninfected strain and four *Wolbachia*-infected *Culex* strains known to differ in their orf7 type (Sanogo and Dobson, 2004) have been compared. Different life stages were examined to characterize orf7 expression throughout mosquito development. Since orf7 is thought to encode a capsid protein (Masui et al., 2000), orf7 expression is expected during virus replication. In addition to transcriptional characterization, electron microscopy was used to observe for exogenic phage particles associated with *Wolbachia*-infected host cell cytoplasm. The results indicate that the *w*Pip infections in *Culex* harbor both actively transcribed virions and inactivated prophages. The expression pattern of orf7 was observed to vary between females and males, between *Culex* strains and between life stages. We discuss the results in relation to the role of the WO phage in the evolution of *Wolbachia* and the potential impact on *Culex* ecology and evolution.

2. Materials and methods

2.1. Mosquito strain maintenance, PCR, and crosses

Four *Wolbachia*-infected *Culex* strains were used: Kunu (KU), Crisse (CR), and *Cx. quinquefasciatus* (CQ) are as previously described (Guillemaud et al., 1997; Sanogo and Dobson, 2004). The Kentucky (KY) strain was established from field-collected egg rafts in Lexington, KY. An uninfected strain (LNT) generated by tetracycline treatment was used as a negative control (Rasgon and Scott, 2003). Larvae used in RT-PCR assays were reared at 14 °C to slow development and facilitate staging of mosquitoes.

For crossing assays, reciprocal crosses were conducted between KY individuals and the KU, CR, and CQ strains. The methods and crossing types of strains KU, CR, and CQ have been previously reported (Sanogo et al., 2005). In brief, 10 virgin female and 10 male mosquitoes were mated, blood-fed with chicks and allowed to oviposit. The number of hatched and unhatched eggs were counted.

As confirmation of the *Wolbachia* and orf7 type, genomic DNA was isolated from the fourth larval instar of each strain using DNeasy kits (QIAGEN Inc., Valencia, USA). The wsp gene and phage WO orf7 variants were amplified as previously described (Sanogo and Dobson, 2004).

2.2. RNA extraction and RT-PCR

Total RNA was isolated from eggs, larvae, pupae, and 2-day-old adults. RNA was extracted from individuals for each life stage except eggs and first instar larvae. To obtain sufficient sample sizes for the latter, egg rafts of approximately 100 eggs and pools of 10 sibling first instar larvae were used.

As a precaution against RNA degradation, materials and reagents were treated with diethyl pyrocarbonate (DEPC; Sigma, St. Louis, MO, USA) and the working area was cleaned with RNaseZap spray (Ambion Inc., Woodward St. Austin, TX, USA). RNA was extracted using an RNeasy kit (QIAGEN Inc., Valencia, USA) according to the manufacturer's instructions. DNA was removed from samples using the TURBO DNA-free kit (Ambion) as per the manufacturer's instructions.

Reverse transcriptase reactions were performed in a PTC-200 thermal cycler (MJ Research Inc., Waltham, MA, USA) using the Platinium RT-PCR thermoscript one-step system (Invitrogen, Carlsbad, USA) and the following conditions: cDNA synthesis: 42 °C for 20 min; PCR: 94 °C for 4 min, and a total of 34 cycles of 1 min at 94 °C, 1 min at the T_m (melting temperature for each primer set shown below), 1 min at 72 °C, and an extension step of 10 min at 72 °C. Primer T_m were 55 °C for primers wsp81F and wsp691R (Zhou et al., 1998), 55 °C for primers orf7-Af/orf7-Ar and orf7-Cf/orf7Cr (Sanogo and Dobson, 2004) and 60 °C for primers orf7-Bf/orf7-Br (Sanogo and Dobson, 2004) and phgWOf and phgWOr (Masui et al., 2000). As an additional test for orf7b transcription, two additional primers [orf7bF2: 5′-TTT KTG CAG CTA ATA GCA AGA; orf7bR2, 5′-CTA GGT CAT CAA TTT CTA GGY CAG] were designed based upon previously published sequences (Sanogo and Dobson, 2004). In all reactions, 20–50 ng of RNA template was used. At least two individuals were tested to represent each strain and life stage. Three or more individuals were tested if results were ambiguous. Amplification products were run on a 1% TBE agarose gel and photographed under UV illumination. As previously reported (Sanogo and Dobson, 2004), the

expected sizes are: wsp, 600 bp; orf7, 400 bp; orf7a, 196 bp; orf7b, 320 bp; orf7c, 296 bp. To examine for false positives that may result from amplification of DNA, a control was included that received 1 U of Platinium Taq polymerase (Invitrogen) instead of the Platinum Taq/reverse transcriptase mixture. The latter control was included for each primer set and mosquito life stage. All of the latter controls were negative (data not shown).

2.3. Isolation and purification of phage particles

Mosquito ovaries from 50 gravid CR and LNT females were dissected under a stereo microscope in phosphate buffer saline (PBS; Gibco, BRL, Grand Island, NY, USA). Dissected ovaries were rinsed three times in PBS and then homogenized in PBS. The homogenate was centrifuged at 250 *g* for 5 min to remove large debris. The supernatant was collected and filtered through a 0.45 μm membrane filter (#191-2045; Whatman Inc., Clifton, NJ, USA). The filtrate was overlayed onto 3 ml of 30% (v/v) sucrose/PBS cushion in a quick-seal polyallomer ultracentrifugation tube (part# 342413; Beckman Instruments, Palo Alto, CA, USA). The tube was then filled with PBS and sealed. Centrifugation was performed using a Beckman Optima XL ultracentrifuge at 220 kG for 1 h at 4 °C using a Ti90 fixed rotor (Beckman). The supernatant was discarded and the resulting pellet was washed three times in 12 ml of PBS by centrifugation at 25 kG for 30 min to remove sucrose.

2.4. Transmission electron microscopy

For negative staining, the pellet resulting from ultracentrifugation was re-suspended in 300 μl of PBS. About 5 μl of the purified phage suspension was placed on a copper mesh formvar carbon-coated grid for 1.5 min. Filter paper was used to remove excess liquid from the sample, which was subsequently negatively stained with a drop of 2% uranyl acetate and air-dried. The samples were examined with a Philip Tecnai Biotwin 12 transmission electron microscope (FEI, Eindhoven, Netherlands).

3. Results

Unlike the other *Culex* strains used in this study, KY has not been previously described. PCR of the KY strain using the wsp general primers (Zhou et al., 1998) demonstrates it to be infected with *Wolbachia* (Fig. 1). Subsequent wsp gene sequencing of the KY infection demonstrated the infection to be *w*Pip *Wolbachia* (AF301010), identical to the KU, CR, and CQ strains. PCR amplification of KY with the orf7 primer sets (Sanogo and Dobson, 2004) resulted in orf7a, orf7b, and orf7c amplification products (Fig. 1). Thus, the KY orf7 DNA amplification pattern is similar to that of the KU and CQ strains. In contrast, the CR strain amplifies using the orf7a and orf7c primer sets only (Fig. 1). The aposymbiotic LNT strain does not amplify with the wsp or orf7 primers. Results from crosses between KY individuals and the other *Wolbachia*-infected mosquito strains are summarized in Table 1. KY females are compatible with CR males and weakly compatible with CQ and KU males. KY males are weakly compatible with KU females and incompatible with CQ and CR females.

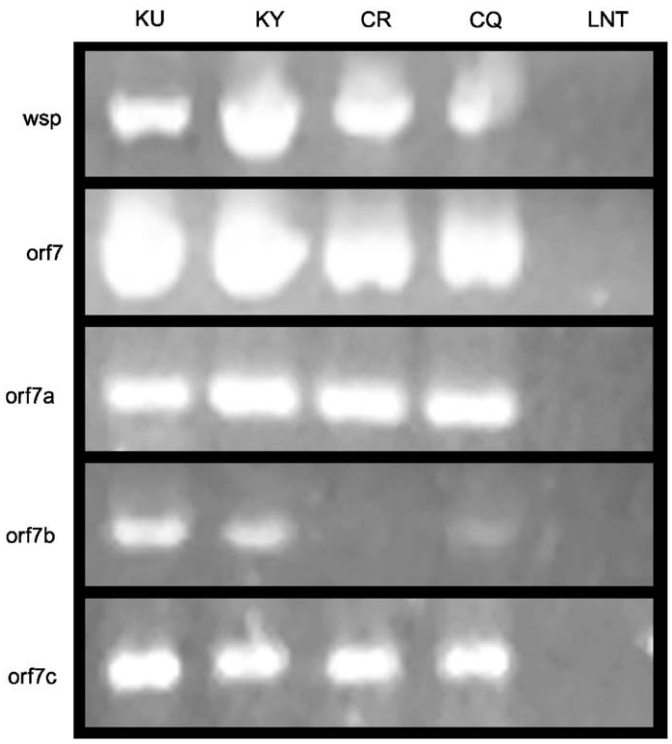

Fig. 1. Detection of *Wolbachia* and phage WO DNA in L4 larval stages of *Culex* strains.

As shown in Fig. 2, wsp is consistently expressed in all of the *Wolbachia*-infected mosquito strains throughout the development. In contrast, expression of orf7 was observed to vary between the *Culex* strains and throughout mosquito development. Notably, no evidence for orf7b transcription was observed. RT-PCR assays failed to detect orf7b transcription in all of the examined mosquito strains and developmental stages. To test the hypothesis that an inability to detect orf7b transcription is due to the larger size of the resulting amplicon (320 bp), an additional primer set (orf7bF2, orf7bR2) was tested with a subset of mosquito samples. Similar to the earlier test, PCR amplification of DNA resulted in amplification products of the expected size (196 bp), but no amplicon was observed in amplification of RNA from KY or CR adults and larvae.

Expression of orf7 in the CR strain was constant throughout the development as measured using the general orf7 primers (Fig. 2). Specifically, orf7 expression was detected in CR eggs, all larval stages, pupae, adult females and males. The weak amplification product from the L2 stage may reflect the relatively small amount of tissue relative to the other samples. In contrast to other samples consisting of larger larvae, an egg raft, or pool of L1 larvae,

Table 1
Egg hatch rates resulting from crosses of *Culex* strains

Females	Males			
	CQ	CR	KU	KY
CQ	*81.0±6.5; 5*[a]	*60.4±10.4; 14*	*74.2±6.8; 12*	13.4; 2
CR	*77.6±9.6; 10*	*75.5±9.6; 9*	*72.4±9.2; 9*	8.9±13.8; 7
KU	*43.7±16.3; 5*	*42.1±36.0; 2*	*80.5±6.0; 2*	45.9±5.9; 4
KY	45.8±28.8; 4	72.4±12.4; 4	24.3±24.4; 3	89.9; 1

Italicized data have been previously reported (Sanogo et al., 2005) and are shown here for comparison.

[a]Percent egg hatch±standard deviation; number of egg rafts per cross.

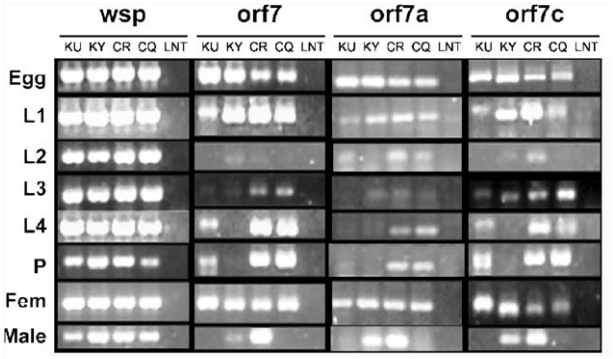

Fig. 2. Detection of *Wolbachia* and phage WO transcripts by RT-PCR reactions in different life stages of *Culex* strains.

only a single L2 larvae was assayed. A similar result was obtained using the diagnostic orf7a and orf7c primer sets (Fig. 2).

The orf7 expression pattern in the KU and CQ strains differs from CR in that expression was not detected in adult males. This pattern was consistent using the general orf7, orf7a, and orf7c primer sets. The KU strain also differs from CR in that it fails to amplify with the orf7a primers in L3 and L4 stages and that a relatively weak orf7a amplification product is observed with pupae (Fig. 2).

Transcription of orf7 in the KY strain is generally similar to the CR expression pattern, with the exception that expression is weak or absent from older larvae and the pupal stage. Expression is detected in eggs, early larvae and adult males and females. This pattern is repeated with the general orf7 primer set and the specific orf7a and orf7c primer sets.

Phage-like particles can be observed in preparations of CR ovaries (Fig. 3). The size of the particles recovered ranged between 40 and 50 nm. The particles have an icosahedral capsid. Although most of the phage particles were observed to be without tails, some were observed with tail-like rudiments or complete tails (15 nm long; including the basic element and fibers; Fig. 3). The particles were not observed in identical preparations of the uninfected LNT strain.

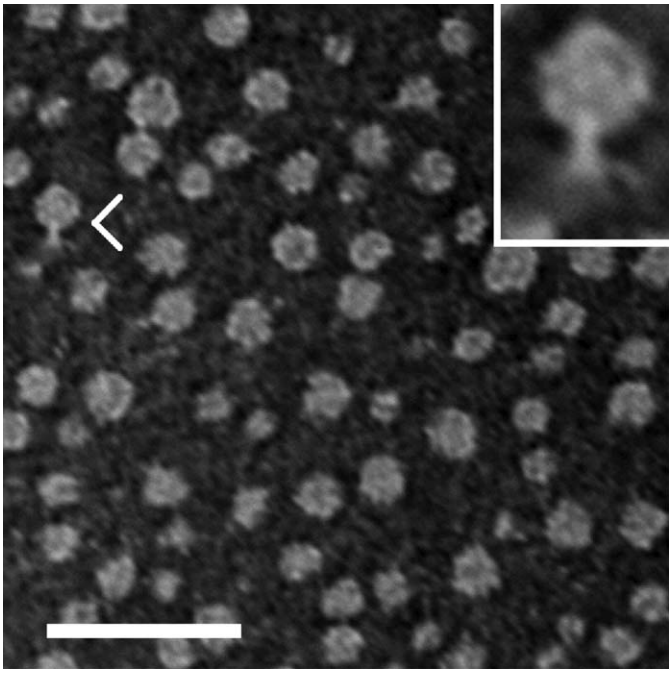

Fig. 3. Electron micrograph of phage WO isolated from the *Wolbachia*-infected CR strain. The white arrow indicates a phage-like particle with complete tail structure (enlarged in the inset). Scale bar = 100 nm.

4. Discussion

The hypothesized activity of the WO phage associated with the *w*Pip infection in *Culex* is supported by observations of orf7 transcription and phage particles. The absence of orf7 RNA and DNA and phage particles from the aposymbiotic LNT strain supports the hypothesis that WO phage is obligately associated with *Wolbachia* infections in *Culex*.

In contrast with orf7a and orf7c transcription, transcription of orf7b was not observed in any of the examined strains at any stage of development, suggesting that orf7b represents an inactive prophage element. Inactivity of orf7b is consistent with the observed inability to PCR amplify orf7b DNA in the CR strain (Fig. 1). The latter reflects a previously identified 5′ end truncation of orf7b in the CR strain (Sanogo and Dobson, 2004). Consistent with the prior report, orf7b DNA was detected in the KU, KY, and CQ strains by PCR.

The observed absence of orf7 transcripts in adult KU and CQ males suggests that phage WO expression is differentially regulated in *Culex* males and females. This is similar to the recent report of sex-specific expression of the WO phage-associated *pk2* ankyrin gene (Sinkins et al., 2005). Expression of orf7 was observed in both females and males in the prior report, which is consistent with observations of the CR strain reported here. Sex- and strain-specific phage activity is consistent with expectations for phage involvement in *Culex* CI. Our results demonstrate that variable expression of WO phage loci is not limited to *pk2* but include orf7 loci, which are thought to

encode structural genes (capsid proteins) and are not expected to be directly involved in CI. Future studies should include examining stage-specific expression of ankyrin loci and experiments examining for tissue-specific transcription of phage loci. A question to be addressed would be whether the female-only transcription reflects expression specific to ovaries and/or the oocytes therein. Additional experiments could examine whether variable orf7 expression corresponds to differences in phage or host type.

Although variation in the observed levels of RT-PCR amplification products observed in Fig. 2 is suggestive of variable stage-specific transcription levels, we emphasize that the RT-PCR technique used in this study is not quantitative. It is likely that differences in primer sensitivity affected the level of amplification product obtained. It should also be noted that in the L2, L3, L4, and pupae, only single individuals were used, whereas pools of eggs and L1 larval instars were used. Thus, the results presented here should be interpreted as a qualitative examination of sex-specific differences and variable expression between *Culex* strains.

Most of the isolated phage particles were without tails. However, some appeared to have tail-like structures. In prior studies, phage tails were not reported (Fujii et al., 2004; Masui et al., 2000; Wright et al., 1978). Sequencing data from *E. kuehniella* (Masui et al., 2000) and *Cadra cautella* (Fujii et al., 2004) infections supports the presence of tail structural genes, including the putative short contractile tail sheath protein gene, suggesting that phage WO is a tailed bacteriophage capable of infecting and injecting DNA into *Wolbachia* during transduction. The large number of virions that appear without tails and the variability observed in tail length may represent an artifact of isolation techniques that result in tail destruction, a photographic artifact (tails hidden due to phage orientation), or natural destruction of phage by the host (Weinbauer and Rassoulzadegan, 2004). Alternatively, the observed morphological diversity may reflect multiple virion types associated with *Wolbachia* infection in *Culex*.

Characterization of *Wolbachia* wsp expression was conducted primarily as a positive control to demonstrate template quality. However, this also represents an initial characterization of wsp transcription in vivo. As shown in Fig. 2, wsp was expressed consistently throughout *Culex* development in all strains and in both sexes. This suggests that *Wolbachia* genes are actively expressed throughout the entire *Culex* life cycle.

A prior study failed to demonstrate a correlation between phage WO orf7 and CI crossing pattern in *Cx. pipiens* (Sanogo et al., 2005). However, the prior analysis did not consider variation in orf7 expression. The results presented here demonstrate that similar analyses in the future should consider a quantitative characterization of expression levels of multiple phage loci (including loci that are potential candidates for affecting CI and loci not thought to be directly involved) in both reproductive and somatic tissues. Future examinations of *Wolbachia* and *Culex* evolution and ecology and the potential for phage involvement in cytoplasmic incompatibility should consider variable phage activity.

Acknowledgments

The authors are thankful to Laurent Gavotte for comments, Alyona Tarima and James Mains for their assistance with mosquito rearing, and the Medical Entomology laboratory at the Center of Ecological Entomology, Illinois Natural History Survey for experimental support. This research was supported by a grant from the NIH (NIH-AI-51533). This is publication 05-08-080 of the University of Kentucky Agricultural Experiment Station.

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