Cloning and characterization of a gene encoding phage-related tail protein (PrTP) of endosymbiont *Wolbachia*

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Abstract ***Wolbachia*** **is an obligatory, maternally inherited intracellular bacterium, known to infect a wide range of arthropods. It has been implicated in causing cytoplasmic incompatibility (CI), parthenogenesis, the feminization of genetic males and male-killing in different hosts. However, the molecular mechanisms by which this fastidious bacterium causes these reproductive abnormalities have not yet been determined. In this study, we report on the cloning and characterization of the gene encoding phage-related tail protein (PrTP) from *Wolbachia* in *Drosophila melanogaster* CantonS (*w*MelCS) and from *Wolbachia* in *Drosophila melanogaster* yw67c23 (*w*Mel) by representational difference analysis (RDA) and ligation-mediated PCR (LM-PCR). The functionality of a bipartite nuclear localization signal sequence (NLS) of the gene was also successfully tested in *Drosophila* S2 cells. PrTP expression in various strains of *Wolbachia* was investigated. Our results suggest that PrTP may not induce CI directly. However, the existence of *prtp* provided direct evidence of phage-mediated horizontal gene transfer (HGT) that might play an important role in a variety of reproductive abnormalities of *Wolbachia*.**

Keywords: ***Wolbachia*****, CI, *prtp*, NLS, HGT.**

Wolbachia, a member of the Rickettsiaceae family, is an obligatory intracellular and maternally inherited bacterium known to infect a wide range of arthropods. Indeed, some surveys suggest that around 20%—76% of all insect species may be naturally infected with this agent, making it one of the most ubiquitous endosymbionts described to date[1,2]. Infections with this agent have been associated with various reproductive abnormalities in the hosts, including parthenogenesis in wasps, the feminization of genetic males in an isopod species, male-killing in *Drosophila* and most commonly, the expression of cytoplasmic incompatibility (CI) in a variety of arthropod species[3]. CI expression usually results in embryonic death in crosses in which the male insect parent is infected with a *Wolbachia* strain and the female parent is either uninfected (Unidirectional CI) or infected with a different *Wolbachia* strain (Bidirectional CI). It appears that a *Wolbachia* strain is able to imprint the sperm of insects that it infects through an unknown mechanism and that this imprint is rescued only in eggs that are infected with the same *Wolbachia* strain.

By taking advantage of these various reproductive manipulations, *Wolbachia* is able to efficiently invade host populations without being infectious or moving horizontally between individuals at an appreciable rate. It has been suggested that *Wolbachia* could be used as a vehicle to drive desirable genotypes, e.g. genes that prevent insect disease vectors from transmitting pathogens to humans, livestock, or plants, into wild insect populations[4,5].

Although much is known about the phenomenology and population genetics of *Wolbachia* infections, we have very little knowledge about the molecular mechanisms for the interaction between this agent and its host. Less molecular knowledge on *Wolbachia* and the inability to grow it on defined cell-free medium have been the major factors underlying these uncertainties. In this study, the genomic difference between *Wolbachia* with different CI phenotypes was analyzed by representational difference analysis (RDA)[6]. A full-length sequence for the gene potentially encoding phage-related tail protein was isolated from *Wolbachia* in *Drosophila melanogaster* CantonS (*w*MelCS) and from *Wolbachia* in *Drosophila melanogaster* yw67c23 (*w*Mel). Subsequently, the characteristics of the DNA sequences and the corresponding putative amino acid products were also investigated. Then, the copy numbers and expression profiles of the gene were determined in different *Wolbachia* strains. In addition, the functionality of a bipartite nuclear localization signal sequence (NLS) of the gene was successfully tested in *Drosophila* S2 cells.

1 Materials and methods

(i) *Wolbachia* and *Drosophila* strains. The following strains of *Drosophila* species which harbor *Wolbachia* infections were used: *Drosophila simulans* Riverside(DSR), *Drosophila simulans* DSW/Mau (Mau), *Drosophila simulans* Noumea(R3A), *Drosophila melanogaster* CantonS(DM), *Drosophila melanogaster*yw 67c23 (YW). The corresponding *Wolbachia* were named *w*Ri, *w*Ma, *w*No, *w*MelCS, *w*Mel, respectively. Of the various *Wolbachia* strains, *w*Ri, *w*No, *w*Mel have been reported as strong CI expressors, while *w*Ma, *w*MelCS as being incapable of expression of the CI phenotype[7]. All of the above flies, devoid of *Wolbachia* by tetracycline treatment, were correspondingly abbreviated to DSRT, MauT, R3AT, DMT and YWT (provided by Prof. Scott O'Neill in Queensland University, Australia).

(ii) Gene cloning. RDA analysis was performed according to protocol[6]. DSW/DSR harboring CI-inducing *w*Ri was used as a tester, while DSW/Mau with non-CI-inducing *w*Ma was used as a driver. The RDA products were then cloned into vector pGEM-T (Promega) and sequenced, followed by BLASTX algorithm analysis[8]. For the DNA fragment showing high homology to a

known gene encoding the PrTP, its flanking sequences were obtained by ligation-mediated PCR(LM-PCR) from the flies mentioned above[9].

(iii) Preparation of total nucleic acids from *Drosophila*. About 100 adult *Drosophila* were collected and stored at −70℃ until use. The flies were frozen in liquid N_2 (for easier tissue breakage), and ground to a fine powder in a mortar by a pestle. The powder was transferred to a Dounce homogenizer containing 2—3 volumes of Holmes-Bonner buffer (0.1 mol/L Tris-HCl, pH 7.5; 0.35 mol/L NaCl; 10 mmol/L EDTA, pH 8.0; 2% SDS; 7 mol/L Urea) and homogenized with some vigorous shakings. DNA was purified by phenol/chloroform extraction until no interphase appeared, followed by ethanol precipitation, phenol/chloroform extraction for the second time and finally resuspension in TE buffer (10 mmol/L Tris-HCl, pH 8.0; 1 mmol/L EDTA, pH 8.0).

(iv) Southern blotting. To determine the copy numbers of the gene, 10 μg of total fly DNAs from different *Drosophila* strains mentioned above were digested with *Eco*R I (no corresponding restriction site in *prtp*). The DNA was transferred to a Hybond™-N+ nucleic acid transfer membranes (Amersham) by upward capillary transfer after separation on a 1% agarose gel. The membrane was baked for 2 h at 80℃. Prehybridization was done at 42℃ for 2 h with prehybridization solution (6×SSC, 5×Denhardt, 0.5% SDS, 100 μg/mL denatured salmon sperm DNA, 50% formamide). The probe spanning the coding region of *prtp* was obtained by PCR with primers 11-Orf-F/11-Orf-R (11-Orf-F: 5′-GGAATTCAAGGCAATAGAGAAACCGAG; 11-Orf-R: 5′-CCGCTCGAGTCAATAAAATGTCTCTAC) and labeled with [α-^{32}P] dATP, using a random-primed DNA labeling kit (Boehringer Mannheim). The labeled probe was added directly to the prehybridization solution and left overnight for hybridization. The membrane was washed with low stringency buffer (2×SSC, 0.1% SDS) for 30 min at room temperature, then twice in high-stringency buffer (0.1×SSC, 0.5% SDS) for 20 min, both at 68℃ and subsequently exposed to Fuji medical X-ray film at -70℃ for 24 h.

(v) Primers for identifying the quality of DNA. The fragment defined by primer pairs of RP49 (RP49-F: 5′-ATGACCATCCGCCCAGCATAC; RP49-R: 5′-CTTGGAGCGCTCGACAATCTC)[10] was used as a positive control, while the universal primers of *Wolbachia* 81F/691R (81F 5′-TGGTCCAATAAGTGATGAAG AAAC; 691R 5′-AAAAATTAAACGCTACTCCA)[7] was used to detect the existence of *Wolbachia* in the genome DNA. The PCR was performed at 94℃, 55℃ and 72℃ 1 min each, repeated for 35 cycles in a buffer containing 2.5 mmol/L $MgCl_2$, 0.25 mmol/L dNTP, and 500 nmol/L of each primer.

(vi) Preparation of RNA from adult flies. Total RNA was extracted from about 100 adult flies using the RNAeX Reagent (Watson), then purified by RNase-free DNase I (Takara) for further study.

(vii) RT-PCR. In a final volume of 5 μL of the primer/template mixture experimental RNA (up to 1 μg/reaction); random primer (0.5 μg/reaction) and nuclease-free water were thermally denatured at 70℃ for 5 min and chilled on ice. The reverse transcription reaction mix was prepared by combining the following components of the ImProm-II™ Reverse Transcription System (Promega) in a sterile 1.5 mL microcentrifuge tube on ice with a final volume of 15.0 μL: nuclease-free water; ImProm-II™ 5×reaction buffer; 1.5—8.0 mmol/L $MgCl_2$; 0.5 mmol/L dNTP; 20 u recombinant RNasin ® ribonuclease inhibitor (optional); 1.0 μL of ImProm-II™ reverse transcriptase.

The mixture of both 5 μL of primer/template mix and 15 μL of the Reverse Transcription Reaction Mix to each reaction was annealed at 25℃ for 5 min, extended at 42℃ for up to 1 h, and blocked at 70℃ for 15 min. 1 μL product of RT reaction was PCR amplified with the primers 11-Orf-F/11-Orf-R. The PCR analysis involved denaturation for 5 min at 94℃, followed by 30 cycles of denaturation at 94℃ for 1 min, annealing at 60℃ for 1 min and extension at 72℃ for 1 min, then final extension at 72℃ for 10 min.

(viii) Functional testing of the NLS region of *prtp* in S2 cell

(1) Construction of green fluorescent protein pEGFP-C/11NLS. The fragment containing the NLS sequence of *prtp* was PCR amplified by using specific primers with incorporated *Eco*R I and *Bam*H I (New England Biolab) restriction sites: 11-G-F: 5′-GGAATTCCAAGGCAATAGAGAAACCGAG; 11-G-R: 5′-CGGGATCCACCAAGTTGACCACCACTTG. To introduce the additional restriction sites to *prtp* sequence, this amplification product was doubly digested with *Eco*R I and *Bam*H I and ligated into the vector pEGFP-C1 (Clontech Laboratories Inc.). The construct was verified by sequencing.

(2) Cell culture and transfection. S2 cell line (provided by Prof. Xu Tian, Yale University in USA) was maintained at 22—24℃ and grown in complete M3 media with 1× standard M3 media (pre-mix powder available from JRH Bioscience), 12.5% fetal calf serum (FCS, Hyclone), 1×penicillin-streptomycin-fungizone (100×stock from JRH Bioscience), and 1×BPYE (5×stock is 12.5 g DIFCO Bacto-peptone, 5 g DIFCO TC-yeastolate per liter water). The day before transfection, 1×10^6 S2 cells were seeded in each 60 mm plate in 4 mL complete M3 media.

The media was exchanged for fresh complete media lacking antibiotics 3 h before transfection, and the cells were transfected with 10 μg of plasmid DNA and a calcium phosphate transfection suspension (Life Technologies) for 24 h according to the manufacturer's instructions. After a change to complete medium, the cells were grown for a further 24 h before processing for microscopy. GFP fluorescence was viewed at 507 nm using a confocal microscope (LEICA TCSNT) with excitation maximum at 488 nmol/L. A series of optical scans through the z axis of the cells was captured and processed.

2 Results

The RDA method has been extensively applied since it was developed in 1993. Researchers have been able to identify cancer-related genes or markers by this method. Since *Wolbachia* cannot be cultured on defined cell-free medium, the genomic difference of *Wolbachia* with different CI phenotypes was analyzed by RDA: DSW/DSR harboring CI-inducing *w*Ri was taken as a tester, while DSW/Mau with non-CI-inducing *w*Ma was used as a driver. In this study, we successfully isolated several DNA fragments, the RDA products were then cloned into pGEM-T vector (Promega) and sequenced. After that, the sequences were analyzed by BLASTX algorithm. Among these fragments, the one showing high homology to a known gene encoding phage-related tail protein (PrTP) was of interest; its flanking sequence was obtained by LM-PCR .

(i) Characterization of DNA sequences and their putative products. A DNA fragment of about 2.2 kb was obtained after LM-PCR. To exclude the error introduced by *Taq* polymerase, three independent colonies were sequenced and the consensus sequence was determined. Moreover, the corresponding amino acid sequences of the open reading frame (ORF) were used to search databases using BlastP, and multiple alignments were performed with ClustalW analysis for functional characterization. The full-length 2205 base pairs (bp) for *prtp* from *w*MelCS (the GenBank accession number: AY043263) contained 5′ and 3′-flanking regions and an ORF coding for 680 amino acids (aa) with a predicted molecular mass of 74699 dalton and an isoelectric point (PI) of 9.34. While the homologous gene (the GenBank accession number: AF420275) from *w*Mel, with 99% identity to the former, also predicted a protein of 680 amino acid residues (molecular mass of 74582 dalton, PI of 9.40). The different amino acid residues between PrTP from *w*Mel and *w*MelCS are shown in table 1. Their G+C content is 36% and they all have strong A/T biased codon usage.

Pfam HMM search (http://pfam.wustl.edu/hmmsearch.shtml) showed that PrTP contained four important functional domains: GlutR (19—134 aa), which uses NADPH as a cofactor[11]; SOR_SNZ (135—249 aa), members of this family are enzymes involved in a new pathway of pyridoxine/pyridoxal 5-phosphate biosynthesis[12]; RTX (250—623 aa), which belongs to a family of bacterial toxins (cytolysins and cytotoxins). This Pfam family represents the N-terminal domain which is found in association with a glycine-rich repeat domain and hemolysinCabind[13]; phosphorylase (295—308 aa). Results from SMART (http://smart.embl-heidelberg.de/) indicated that there were three transmembrane regions in PrTP: 440—462 aa, 474—493 aa, 497—519 aa. Moreover, Methyl-accepting chemotaxis-like domains (chemotaxis sensory transducer) (114—339 aa) is thought to undergo reversible methylation in response to attractants or repellants during bacterial chemotaxis.

Table 1 Different amino acids (aa) between PrTP from *w*MelCS (AY043263) and *w*Mel (AF420275)

AA position site	PrTP (AY043263)	PrTP (AF420275)
187	F	S
198	I	V
341	F	L
371	E	K
490	A	V
520	T	A
583	L	H
678	T	A

Basic domains are well known to functions as NLS[14]. Motifscan Analysis (http://hits.isb-sib.ch) of the N-terminal domains of PrTP revealed that two basic regions (letter in bold) (**KR**DALHSFNEEV**RK**SGRN) (32—49 aa) together resemble the bipartite NLS, consisting of two short stretches of basic amino acids separated by 10—22 residues (fig. 1). Moreover, the result from ScanProsite (http://us.expasy.org/cgi-bin/scanprosite) shows that there are 2 leucine zipper patterns in the PrTP:

1. LVSDQHKLGSSIEVLKGKYGKL(53—74 aa);
2. LKV VAVGLGYGFTLLGSTIFSL (451—472 aa).

PrTP	32KRDALHSFNEEVRKSGRN49
Nucleoplasmin	559KRPAATKKAGQAKKKK595
pRb	561KRSAEGSNPPKPLKKLR577
c-Jun	259KRMRNRLAASKCRKRK274
p53	305KRALPNNTSSSPQPKKK321

Fig. 1. The basic domain of PrTP. Two basic domains of PrTP together resemble the bipartite NLS of nucleoplasmin and other proteins, including pRb, c-Jun and p53.

It is well known that the N-terminal basic region plus leucine zipper like domain was the DNA-binding characteristic domain[15]. Considering the reproductive abnormalities of the host caused by *Wolbachia*, we suggested that PrTP might play a potential role in the interaction between *Wolbachia* and its host.

By BlastP, PrTP shared about 26% identity, 49% similarity to phage-related tail protein XF0730 from *Xylella fastidiosa* (strain 9a5c) (NP_298020,NP_299760);

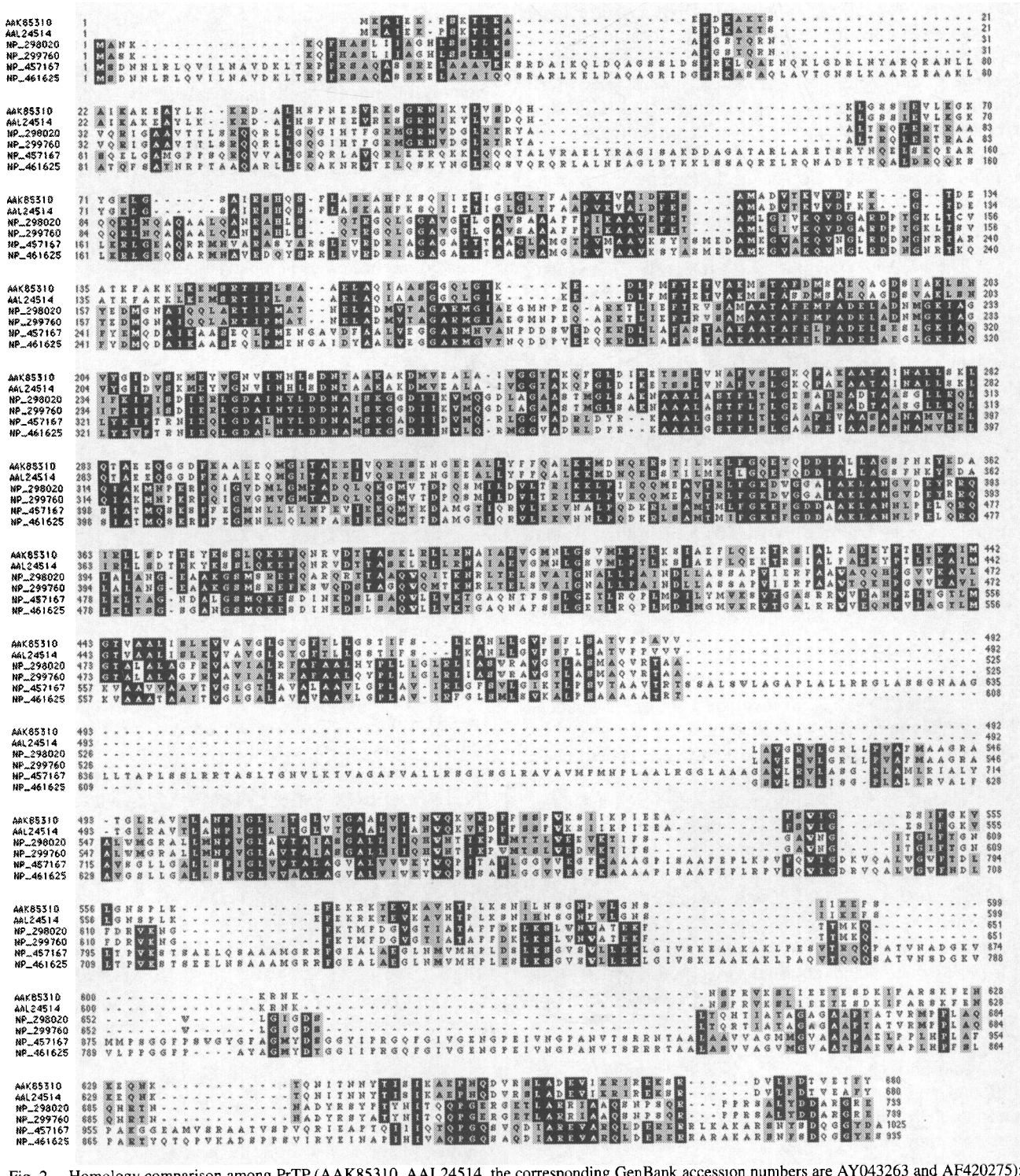

Fig. 2. Homology comparison among PrTP (AAK85310, AAL24514, the corresponding GenBank accession numbers are AY043263 and AF420275); phage-related tail protein from Xylella fastidiosa 9a5c (NP_298020, NP_299760); Fels-2 prophage: similar to orfG protein in phage 186 from Salmonella typhimurium LT2 (NP_461625) and putative bacteriophage tail protein [Salmonella enterica subsp. enterica serovar Typhi] (NP_457167). Alignment was performed at NCBI (http://www.ncbi.nlm.nih.gov) and Clustal W(http://www.ebi.ac.uk). Identical amino acid residues are indicated by dark shading, and conservative substitutions are indicated by light shading.

21% identity, 41% similarity to Fels-2 prophage: similar to orfG protein in phage 186 from *Salmonella typhimurium* LT2 (NP_461625); 23% identity, 43% similarity to putative bacteriophage tail protein from *Salmonella enterica subsp. enterica serovar* Typhi (NP_457167) (fig. 2). It indicated that the origin of the PrTP might be from bacteriophage.

(ii) Molecular analysis of PrTP. To determine the copy number of *prtp* gene in different *Wolbachia* strains, Southern blot was performed by using the full-length DNA sequence of *prtp* as a probe. The results revealed that the genome of *w*Ri contained three DNA fragments (fig. 3), but the full-length of *prtp* from *w*Ri could not be obtained, the bands with strong signal reflected the homologous fragment of *prtp*. *w*Mel and *w*MelCS contained a single gene copy. There was no homology detected from other strains. In this experiment, the fragment defined by primer pairs of RP49[10] was used as a positive control, while the universal primers of *Wolbachia* 81F/691R were used to detect the existence of *Wolbachia* in genomic DNA. Therefore the negative results indicated the non-existence of such an element in the genomes of those strains.

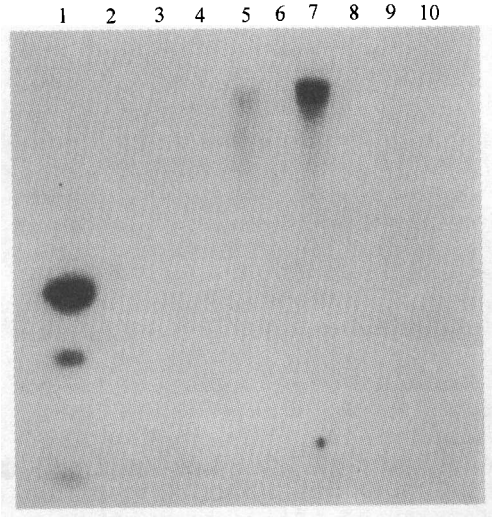

Fig. 3. Southern blot analysis. The total fly DNA digested with *Eco*R I and probed with the full-length DNA of *prtp* from *w*MelCS. 1, DSR; 2, DSRT; 3, R3A; 4, R3AT; 5, YW; 6, YWT; 7, DM; 8, DMT; 9, Mau; 10, MauT.

To investigate the expression of *prtp* in different strains of *Wolbachia*, reverse transcriptase-mediated polymerase chain reaction (RT-PCR) was carried out. The result (fig. 4) was consistent with that from the Southern blot except that no fragment was detected from *w*Ri.

To establish whether these putative NLS domains were responsible for the nuclear import of *Wolbachia*, part of the *prtp* sequence containing NLS region was ligated into vector pEGFP-C1 and transiently transfected into S2 cells. pEGFP-C1 containing the NLS region (pEGFP-C/11NLS) was found to be localized predominantly within the nucleus of all transfected S2 cells (Plate Ⅰ). In contrast, GFP protein alone was distributed evenly throughout the cell. It suggested that NLS may be responsible for the nuclear import of *Wolbachia in vitro*.

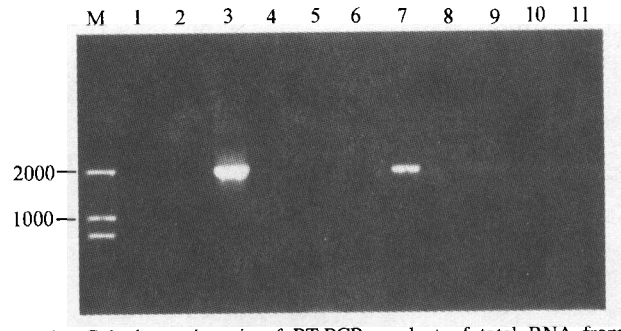

Fig. 4. Gel electrophoresis of RT-PCR product of total RNA from different *Drosophila* strains (digested with RNase-free DNase I) amplified by 11-orf-F/11-orf-R. M, DL2000; 1, DSR; 2, DSRT; 3, YW; 4, YWT; 5, R3A; 6, R3AT; 7, DM; 8, DMT; 9, Mau; 10, MauT; 11, Blank.

3 Discussion

Bacteriophages can mediate the evolution, the transfer of virulence factors and occasional acquisition of new traits by the bacterial host[16]. *prtp* shows high similarity to the phage-related tail gene from the plant pathogen *Xylella fastidiosa*. However, *prtp* shows little homology to the sequences of phage WO, a bacteriophage-like genetic element first discovered in the *Wolbachia* genome[17]. It also shows little homology to the phage-related genes from *Rickettsia conorii* and *Rickettsia Prowazeki*[18], which are related species to *Wolbachia*. The results from BlastP in NCBI showed that PrTP may originate from a dsDNA virus: Caudovirales (http:www.ncbi.nlm.nih.gov/Blast and http://www.ncbi.nlm.nih.gov/Taxonomy), significantly different from phage WO in *Wolbachia* reported before, which presents the direct evidence of extensive horizontal gene transfer (HGT).

Wolbachia cause a variety of reproductive abnormalities, and each *Wolbachia* strain seems to have its own phenotype for the abnormalities[19]. Since these phenotypes do not reflect the *Wolbachia* phylogeny, and are rather randomly distributed among *Wolbachia* strains, it has been postulated that genes for the abnormalities are conveyed by mobile genetic elements such as plasmids and phages[17,19,20]. The cloning and characterization of PrTP in *Wolbachia* demonstrated HGT between *Wolbachia* and phage. In PrTP, its DNA-binding region of the N-terminal basic portion plus leucine zipper-like domain suggests that it can interact with host genomic DNA. Moreover, *in vitro* test showed that NLS of PrTP was localized predominately in the nucleus of S2 cells of *Drosophila*. Our study provides insights into the potential role of PrTP in mediating the binding between *Wolbachia* and the host chromosome, which induce a variety of reproduc-

tive abnormalities on the host.

In the beginning, the fragment of *prtp* from *Wolbachia* with a different CI phenotype was obtained by RDA, and its flanking sequences were obtained by LM-PCR from different *Wolbachia* strains. Combined with the results from Southern and PT-PCR, it suggested that PrTP itself may not be correlated directly in all the processing of CI. For example, all *w*Ri, *w*No, *w*Mel could induce CI but *prtp* (or a fragment of the homologue) was not detected in *w*No genome. *w*MelCS could not induce CI, although it indeed contained *prtp*. The relationship between PrTP and CI is still unknown, further study is required.

The reason why *w*Ri contained three homologous fragments of *prtp* might be explained by the fact that the genome size of *w*Ri was the biggest one found so far among different *Wolbachia* strains, *w*Ri may undergo more gene duplication during its evolutionary process[21]. Unfortunately, the full-length of *prtp* from *w*Ri could not be obtained, the following reasons could underlie this phenomena. Firstly, given that PrTP itself is a phage-related tail protein, the alignment analysis showed that it originates from phage or prophage, maybe the process of HGT is ongoing. Secondly, compared with the infection of other *Wolbachia* strains, *w*Ri infection may be much more recent because it is associated with a specific mitochondria DNA subtype[22–24]. It raised the most possibility that *w*Ri locates the special position in the route for evolution among the host, *Wolbachia* and phage, the process of horizontal gene transfer between *w*Ri and phage, the interaction of *w*Ri and host are all on the way.

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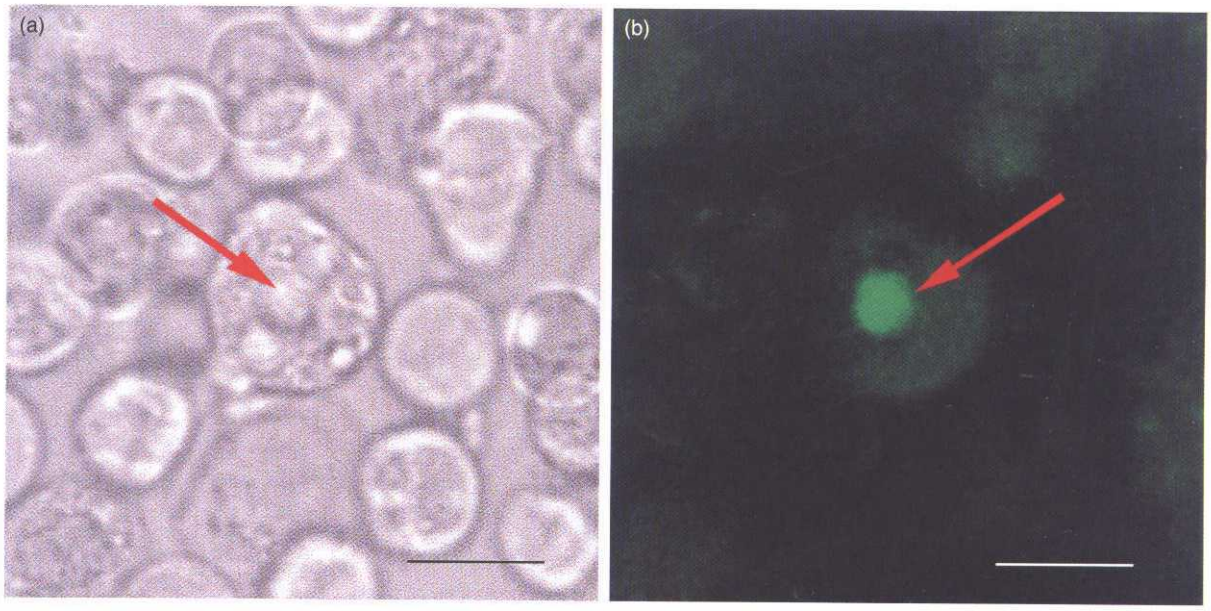

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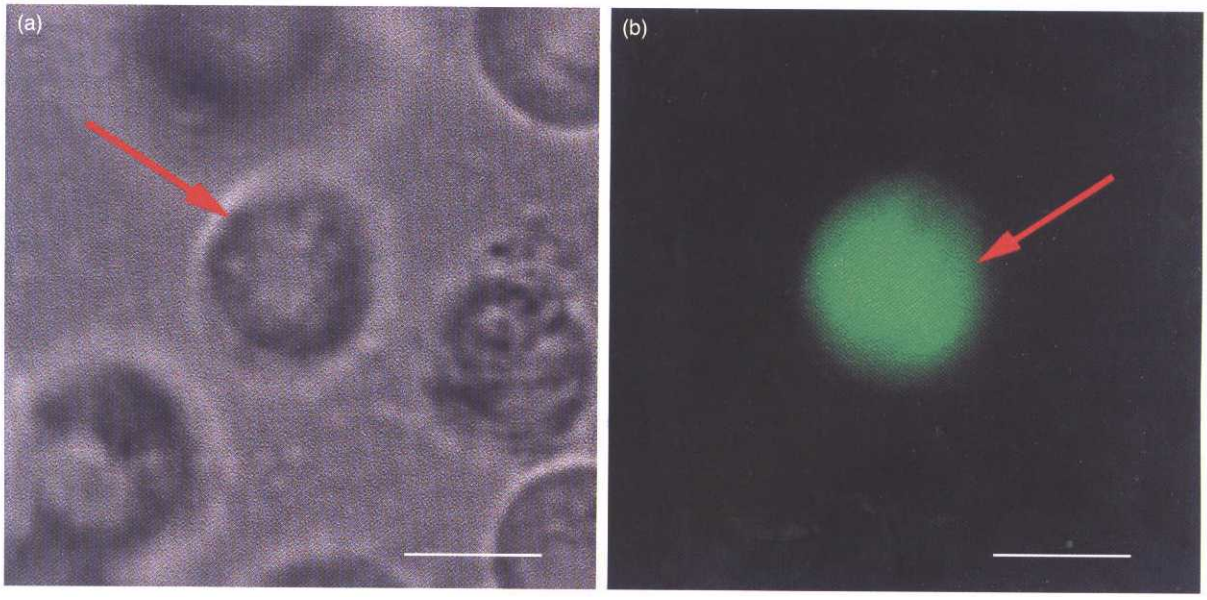

2

Intracellular distributions of EGFP in transfected S2 cells. The fluorescence localization of EGFP/11NLS (1), EGFP(2) was compared in transiently translected S2 cells. EGFP proteins were expressed for 48 h, and their distribution was examined by confocal microscope with amplification ×500. EGPF/11NLS is present primarily in the nucleus of transfected cells, whereas EGFP alone diffuses through out the cell. Bars: 10 μm. 1(a) Under light vision, 1(b) under fluorescence vision, red arrow indicates the nuclear of the cell. 2(a) Under light vision, 2(b) under fluorescence vision, red arrow indicates the whole cell.