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Characterization and transcriptional analysis of two gene clusters for type IV secretion machinery in *Wolbachia* of *Armadillidium vulgare*

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Abstract

Wolbachia are maternally inherited α-proteobacteria that induce feminization of genetic males in most terrestrial crustacean isopods. Two clusters of *vir* genes for a type IV secretion machinery have been identified at two separate loci and characterized for the first time in a feminizing *Wolbachia*. Furthermore, we demonstrated that these operons are transcriptionally active in ovaries and in all other tissues tested, suggesting that T4SS has a significant role in *Wolbachia* biology. These observations and the identification of homologous *vir* genes in *Wolbachia* strains infecting insects or nematodes show that *vir* genes are conserved among *Wolbachia* strains whatever the phenotype induced by the bacteria.

Keywords: Crustacean; Isopod; *Wolbachia*; Feminization; Cytoplasmic incompatibility; T4SS; *vir* Genes

1. Introduction

Bacteria belonging to the *Wolbachia* genus have been recognized to infect a wide range of arthropods and some nematodes [16]. *Wolbachia* are maternally inherited intracellular bacteria that remain difficult to culture. These Gram-negative bacteria belong to the group of α-proteobacteria and its closest known relative groups are *Rickettsia*-like bacteria.

Wolbachia are able to invade and maintain themselves in numerous arthropod host species by inducing a variety of reproductive alterations including male feminization in isopod crustacean species [17], parthenogenetic development in wasps or mites, male-killing in insect species and cytoplasmic incompatibility (CI) in isopod or insect species [5]. To date, nothing is known about bacterial and host genes involved in induction of these reproductive alterations, and molecular mechanisms have not yet been elucidated.

Feminization is a phenomenon whereby infected genetic males are converted into functional females by inhibition of androgenic gland development [17]. Since *Wolbachia* are exclusively localized in the host cell cytoplasm, it is believed that the bacterium secretes macromolecules in order to reverse sexual differentiation. Many Gram-negative bacteria such as *Agrobacterium tumefaciens*, *Helicobacter pylori*, *Bordetella pertussis*, *Legionella pneumophila*, *Brucella* spp. or *Bartonella* spp. use a type IV secretion system (T4SS) to deliver virulence factors within the cytoplasm of host cells [8]. Amongst obligate intracellular bacteria, T4SS has been found previously in *Rickettsia prowazekii* [1] and characterized in *Rickettsia*-like bacteria such as *Anaplasma phagocytophila* and *Ehrlichia chaffeensis*, where eight *virB* and *virD* genes were clustered at two separate loci and polycistronically transcribed [15]. Furthermore, five genes coding for the T4SS (*virB8*, *virB9*, *virB10*, *virB11* and *virD4*) were identified in *w*Tai and in *w*KueYO, which are *Wolbachia* symbionts of the Taiwan cricket and the Mediterranean flour moth, respectively [13]. More recently, the complete genome sequence of different *Wolbachia* strains infecting *Drosophila melanogaster* (*w*Mel)

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and *Drosophila ananassae* (*w*Ana), or the nematode *Brugia malayi* (*w*Bm) [10], also revealed complete T4SS as two operons as in *Anaplasma* and *Ehrlichia* [15].

Amongst *Wolbachia*, T4SS has only been identified in the CI *Wolbachia* of insects and in the mutualistic *Wolbachia* of nematodes. Therefore, in this study, we checked for the presence of the T4SS in isopod *Wolbachia* strains that induce another phenotype, the feminizing *w*Vul, *w*Ase and *w*Pru harbored by *Armadillidium vulgare*, *Oniscus asellus* and *Porcellionides pruinosus*, respectively [17]. The presence of T4SS in a CI *Wolbachia* of isopod, *w*Pet harbored by *Porcellio dilatatus petiti*, has also been tested.

2. Materials and methods

2.1. Wolbachia strains

Uninfected and *Wolbachia*-infected terrestrial isopods *A. vulgare*, *O. asellus*, *P. dilatatus petiti*, *P. pruinosus* were reared in the laboratory at 20 °C, on moistened soil with dead leaves and carrots as food. *Wolbachia* infection was checked by PCR using specific *wsp* primers (Supplementary data 1).

2.2. Amplification and sequencing of vir genes

DNA extractions were performed from ovaries or testes as described elsewhere [4]. Several degenerated primer pairs were designed based on the conserved sequences of *vir* genes among *A. phagocytophila*, *E. chaffeensis*, *w*Tai, *w*KueYO, *w*Mel, *w*Ana or *w*Bm (Supplementary data 1). The DNA fragments amplified by PCR (40 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min per kb) using these primers were sequenced by a dideoxy termination method using the kit "ABI PRISM™" (Applied Biosystems), according to the manufacturer's instruction, and using the Walking chromosome method for long PCR fragments (ESGS or QIAGEN Companies). The sequence data from this study has been submitted to GenBank under accession numbers AY967767 (*phophatase-virD4*) and AY967766 (*virB3–virB6*). The missing 3′-ends of *virB6* and *virD4* genes were obtained from shotgun library clones (whole-genome shotgun-sequencing project: European *Wolbachia* project EUWOL). A database search was carried out with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

2.3. RNA isolation and transcriptional analysis

Total RNA was isolated from different tissues (ovaries, hematopoietic organs, hemocytes, nerve chains, brains, gut and adipose tissue) using the RNeasy Mini-kit according to the manufacturer's instructions (Qiagen) and reverse-transcribed using a Thermotranscript kit (Invitrogen-Life Technologies) with random hexamer primers at 25 °C for 10 min and at 50 °C for 1 h. PCR was performed under conditions described above using primers as indicated (Fig. 3, Supplementary data 1). Nested PCRs were also realized using internal primers to reveal *vir* gene expression in nerve tissues.

2.4. Genomic Southern blot analysis

Genomic DNA extracted from infected ovaries (20 µg) was digested with restriction endonucleases, electrophoresed and subjected to Southern blotting. The 4.67 kb *virB8–virD4* (virB8-1F/virD4R) and the 2.83 kb *virB3–virB6* (virB3-F/5′virB6-R) PCR fragments were labelled with [α-^{32}P]dCTP by the random primer method and hybridized to nylon membranes. Hybridized blots were imaged and analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

3. Results and discussion

Initially, we successfully amplified the *virD4* gene only in infected ovaries or testes of different isopod species (Supplementary data 2). Hence, based on the conserved sequences of several *vir* genes of different *Wolbachia* strains, several primer pairs were designed and used to amplify DNA fragments corresponding to two *vir* gene clusters (Fig. 1a). The first one, named *virB8–virD4* operon, was composed of five

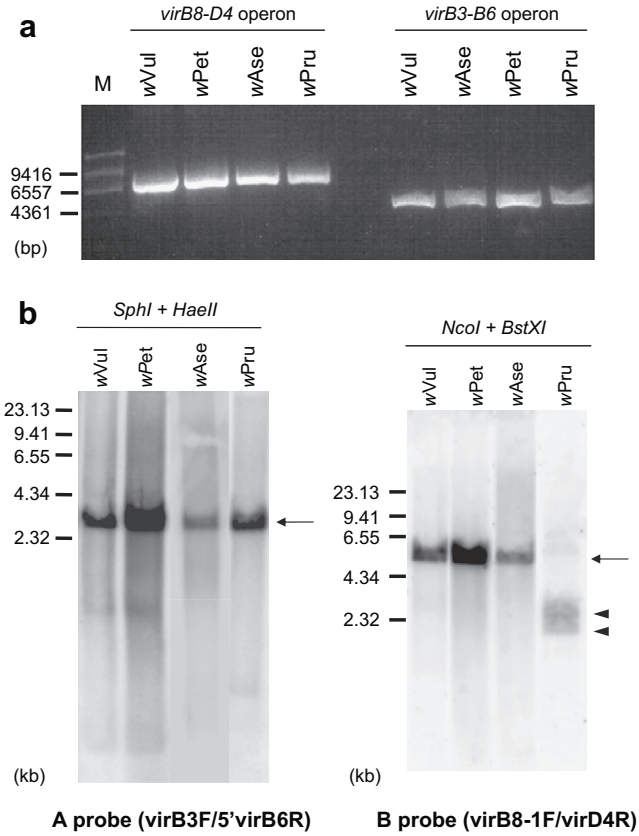

Fig. 1. Detection of *virB* and *virD* genes by PCR and Southern blotting. Detection of *virB3–virB6* and *virB8–virD4* operons by PCR (a) using virB3F/virB6R and phosph-1F/virD4R primer pairs or by Southern blots (b) using A and B probe, respectively, in different *Wolbachia* strains harbored by *A. vulgare* (*w*Vul), *O. asellus* (*w*Ase), *P. pruinosus* (*w*Pru), *P. dilatatus petiti* (*w*Pet). M: DNA size markers. Arrows and arrowheads indicate hybridized fragments.