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Conservation of the Type IV Secretion System throughout *Wolbachia* evolution

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ABSTRACT

The Type IV Secretion System (T4SS) is an efficient pathway with which bacteria can mediate the transfer of DNA and/or proteins to eukaryotic cells. In *Wolbachia pipientis*, a maternally inherited obligate endosymbiont of arthropods and nematodes, two operons of *vir* genes, *vir*B3–B6 and *vir*B8–D4, encoding a T4SS were previously identified and characterized at two separate genomic loci. Using the largest data set of *Wolbachia* strains studied so far, we show that *vir* gene sequence and organization are strictly conserved among 37 *Wolbachia* strains inducing various phenotypes such as cytoplasmic incompatibility, feminization, or oogenesis in their arthropod hosts. In sharp contrast, extensive variation of genomic sequences flanking the *vir*B8–D4 operon suggested its distinct location among *Wolbachia* genomes. Long term conservation of the T4SS may imply maintenance of a functional effector translocation system in *Wolbachia*, thereby suggesting the importance for the T4SS in *Wolbachia* biology and survival inside host cells.

Introduction

Wolbachia pipientis is an unculturable obligate endosymbiont belonging to the α-Proteobacteria (Rickettsiales). They are estimated to be present in ~60% of arthropod species [1,2] and in filarial nematodes [3]. The interactions between *Wolbachia* and their hosts range from pathogenesis to parasitism and mutualism. In many arthropod species *Wolbachia* parasite host reproduction by inducing cytoplasmic incompatibility, feminization, male killing and parthenogenesis to enhance their transmission and spread in hosts [1,4]. Thus, intracellular lifestyle implies communication between *Wolbachia* and their host cells.

Many symbiotic and pathogenic bacteria are able to export DNA and/or proteins into eukaryotic host cells. Such bacteria use a wide range of mechanisms for the transport of proteins and/or DNA across cell membranes, including Type IV Secretion Systems (T4SSs) [5]. T4SSs were considered as effector translocators in several α-Proteobacteria, including *Agrobacterium tumefaciens*, *Bartonella* spp. and *Brucella suis* (Ref. [6]).

In the Rickettsiales order, which mainly comprise obligatory endosymbiotic bacteria of *Rickettsia*, *Ehrlichia*, *Anaplasma* and *Wolbachia* genera, orthologs of T4SS genes are related to those of the *A. tumefaciens vir*B cluster [6]. In contrast to *A. tumefaciens*, Rickettsiales genomes have lost non-essential genes such as those encoding pilus-associated proteins (VirB1, -B2, -B5 and VirB7) involved in host cell attachment, presumably due to intracellular lifestyle [7–11]. However, essential T4SS genes for bacteria biology and survival in host cellular environment are expected to be conserved. Hence, the minimum components of typical and functional T4SS are present in Rickettsiales and consist of *vir*B4, -B8, -B9, -B10, -B11 and *vir*D4 homologs [12]. Unlike *A. tumefaciens*, these genes are clustered in two separate loci in Rickettsiales genomes [8,13,14], including *Wolbachia* (Refs. [7,9,10]). One cluster is composed of five tandem genes (*vir*B8, -B9, -B10, -B11 and *vir*D4) and the other one contains three tandem genes (*vir*B3, -B4 and *vir*B6). Both operons were found to be transcriptionally active in ovaries of different arthropod hosts [11,15–17] but also in all *Wolbachia*-infected tissues from the isopod *Armadillidium vulgare* [15].

The *Wolbachia* T4SS has been suggested to be a potential pathway to export effectors into host cytoplasm and therefore it may be involved in *Wolbachia*-induced host phenotypes [7]. If so, it is expected that the *Wolbachia* T4SS should be conserved at an evolutionary timescale. In this context, we assessed the genomic structure, location and evolution of T4SS loci in a large data set of 37 *Wolbachia* strains belonging to 5 supergroups [18] and encompassing a wide range of known phenotypes.

Materials and methods

Wolbachia strains. Thirty-seven *Wolbachia* strains were used (Table S1). Sequence data available in databases were used for 10 *Wolbachia* strains (Table S1). In addition, total DNA from 27 arthro-

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pod hosts was extracted as previously described [19] (Table S1). Infection status of each individual was checked or tested using a PCR assay based on the *Wolbachia*-specific *wsp* gene, as previously described [20].

DNA amplification and sequencing. T4SS loci structure (*i.e.* gene content and order) was analyzed in the 27 *Wolbachia* strains shown in bold in Table S1 by a PCR strategy amplifying overlapping fragments. Based on available T4SS sequence data from 7 *Wolbachia* strains [7,9,11,15,16], gene orthologs were aligned and specific oligonucleotide primers were designed in conserved regions of the two operons (Table S2). Primers were also designed to amplify *vir*B8–D4 operon flanking regions including the *dna*A, *rib*A and *phos* genes at the 5′ end of *vir*B8 and *wsp*B at the 3′ end of *vir*D4 (Table S2). Strain-specific primers surrounding the *vir*B8–D4 operon of the *w*VulC *Wolbachia* strain infecting the isopod *A. vulgare* were designed using sequences from shotgun library clones of the ongoing EuWol sequencing project (contract #QLK3-CT2000-01079) (Table S2). PCR amplification and DNA sequencing were performed as previously described [20].

Sequence analyses. Full-length *vir*B3, *vir*B11 and partial *wsp* sequences from the 37 *Wolbachia* strains were used to construct phylogenetic trees as previously described [20] (Table S1). To assess the quality of phylogenetic signals, we compared tree topologies based on *vir*B3, *vir*B11 and *wsp* taken individually or concatenated in different combinations.

To test for selected sites, analyses of the *vir* sequences from the studied strains were performed using a local codon model of the web-server http://www.datamonkey.org [21]. Pairwise estimates of the number of non-synonymous (d*N*) and synonymous (d*S*) substitutions per site were compared to the observed mean ω ratio (d*N*/d*S*) using the codeml program in the PAML package v3.14 [22]. The random effects likelihood (REL) analysis [21] was used to identify individual sites of codon alignments under selective pressure based on a Bayes factor of 95.

Results and discussion

Structural organization of T4SS operons is conserved among Wolbachia strains

To gain insight into the role of T4SS in the biology of the unculturable *Wolbachia*, we assessed diversity, genomic location and evolution of T4SS loci in 37 *Wolbachia* strains. Both *vir* gene operons were successfully amplified in our 27 *Wolbachia* strains. Operon size and *vir*B gene order (Fig. 1) were conserved in all *Wolbachia* strains examined [7,9–11,15–17]. *Wolbachia* T4SS loci do not encode all the proteins involved in bacterial conjugation systems from which they may have evolved [6]. Genes homologous to *vir*B1, -B2, -B5 and *vir*B7 of *A. tumefaciens* are not found in *Wolbachia* genomes and the present *vir*B loci may encode the minimum T4SS components of a functional translocator system [12]. The same organization is recorded in *Rickettsia*, *Ehrlichia* and *Anaplasma* relatives of *Wolbachia* (Fig. 2). This observation suggests that T4SS operons structure may have been shared by the common ancestor of all the Rickettsiales [7–11,13,14]. This may be a consequence of genome reduction induced by an intracellular lifestyle of these obligatory endosymbiotic bacteria, as observed in the streamlined genomes of many symbionts such as *Wolbachia* [8].

We also found that all *Wolbachia* T4SS components are highly conserved at the nucleotide level. Nucleotide sequences of *vir*B3 (297 bp) and *vir*B11 (993 bp) were obtained from 27 *Wolbachia* strains and manually aligned with available sequences from 10 additional *Wolbachia* strains (Table S1). *vir*B3 and -B11 gene lengths were conserved apart from three *Wolbachia* strains infecting the nematodes *Brugia malayi* (*w*Bm), *Dirofilaria immitis* (*w*Dm) and the spider *Segestria florentina*, in which *vir*B11 stop codon was observed 9–12 bp downstream relative to other strains. Average nucleotide divergence over all sequence pairs was 0.052 ± 0.008 for *vir*B3 and 0.092 ± 0.006 for *vir*B11. By contrast, *wsp* sequences exhibited two- to fourfold higher average nucleotide divergence (0.192 ± 0.013 hypervariable regions excluded) than *vir*B3 and *vir*B11 among the same *Wolbachia* strains. Phylogenetic reconstructions based on *vir*B3 or *vir*B11 sequences revealed overall congruent information with those based on *wsp* sequences whatever the reconstruction method chosen (data not shown). *Wolbachia* strains were clustered in different supergroups as previously described [18]. No correlation between tree topology and *Wolbachia* extended phenotype could be inferred from our results.

When comparing sequence evolutionary rates independently among *Wolbachia* strains, *vir* genes seem to evolve under strong negative selection. Mean ω = d*N*/d*S* ranged from 0.11 to 0.65 (Table 1). No gene with d*N* > d*S* was inferred from this dataset, suggesting that any site essential for the function of these proteins would evolve under drastic evolutionary pressures. The highest ω ratio corresponds to *vir*B6 which is also the most divergent T4SS gene exhibiting size heterogeneity and ~28% divergence between *Wolbachia* strains. On the contrary, the lowest ω ratio was obtained for *vir*B4. Additionally, *vir*B4, -B11 and *vir*D4 genes exhibited significant negative selection acting on ~20–50% of their sites (Table 1). These three proteins are known to possess ATPase activity and are involved in effector recruitment in other bacteria [5]. Although secretory systems are mostly conserved among bacteria, substrates of the individual systems may differ from each other depending on the specificity of delivered effector(s) [5]. To date, no functional evidence has shown involvement of T4SS in effector transfer in *Wolbachia*. Nevertheless, T4SS has been shown to be responsible for the secretion of an ankyrin repeat-containing protein (AnkA) in *Anaplasma phagocytophilum*, which is closely related to *Wolbachia* [23]. Together with transcriptional analyses demonstrating coexpression of *vir* genes within each cluster [11,15–17], these results suggest a major role for the T4SS in *Wolbachia* biology.

Flanking genomic regions of the virB8–D4 operon are highly variable

Analyses of flanking regions surrounding the *vir*B8–D4 operon revealed different patterns (Figs. 1 and 2). The first gene identified upstream of the *vir*B8–D4 operon, *rib*A, encoded a bifunctional enzyme (3,4-dihydroxy-2-butanone-4-phosphate synthase and GTP cyclohydrolase II), which catalyzes two essential steps in riboflavin biosynthesis [24]. This gene was found upstream of the *vir*B8–D4 operon in all the *Wolbachia* strains, as well as in 5 *Ehrlichia* genomes, 2 *Anaplasma* genomes, and in the *Neorickettsia sennetsu* genome (Ref. [14]). This feature suggests that a *rib*A-*vir*D4 locus may have been present in their common ancestor. In *Anaplasma* and *Ehrlichia*, transcriptional analyses revealed that promoters of the *vir*B8–D4 operon are located within the intergenic spacer between *rib*A and *vir*B8 as well as in the 3′ end of *rib*A [24]. Therefore, conservation of at least *rib*A upstream of *vir*B8 may be important for the integrity and transcriptional activity of the *vir*B8–D4 operon in *Anaplasma*, *Ehrlichia* and *Wolbachia*. Additionally, a Ser/Thr protein phosphatase encoding gene, *phos*, belonging to the calcineurin-phosphoesterase superfamily [25], is also located upstream of *rib*A in all the *Wolbachia* studied, constituting a *phos*–*vir*D4 locus which may have been present since the *Wolbachia* ancestor.

Diverse genes were localized upstream of these two genes (Fig. 1). *dna*A, which is essential in *Escherichia coli* for initiation of bidirectional replication at the chromosomal origin of replication [26], was detected in 15 *Wolbachia* strains, including all A- and G-supergroup *Wolbachia* strains tested, as well as the B-supergroup *w*Sn and the D-supergroup *w*Bm strains. This result is congruent with previous observations [7,9,11,17,26]. Interestingly,