Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium Wolbachia from the novel host Folsomia candida (Hexapoda, Collembola) and its implications for wolbachial taxonomy

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Abstract

Wolbachia pipientis are intracellular, transovarially inherited α-Proteobacteria in invertebrates. Four major Wolbachia groups exist: A, B (contained in divergent arthropods), C and D (harbored by Nematoda). By means of transmission electron microscopy, we observed Wolbachia-like bacteria in a primitive insect, Folsomia candida (Hexapoda, Collembola, Isotomidae). 16S rDNA analysis proved them to constitute a novel lineage, henceforth named group E, in the wolbachial phylogenetic tree. It shares 97.8% 16S rDNA homology with its nearest neighbors, groups A and B, which diverged from it more recently. We propose (i) a new taxon E for the Wolbachia strain in F. candida, (ii) that the single-described Wolbachia pipientis fall apart into at least three species: C, D and the large E-A-B complex. F. candida’s group E Wolbachia rekindle the question about invasive capacities of free-living ancestral wolbachiae and horizontal transfer. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Wolbachia are maternally (= vertically or transovarially) transmitted tiny α-Proteobacteria (Rickettsiales) infecting principally the gonadal tissues of invertebrate hosts. The type species Wolbachia pipientis was described from the gonads of the mosquito Culex pipiens [1,2]. W. pipientis-like bacteria may (i) feminize genetic males [3,4], (ii) enhance host fertility [5] and induce (iii) thelytokous (= mother-to-daughter) parthenogenesis [6–9] and (iv) cytoplasmic in-
compatibility (e.g. [10–14]), each of which results in preferential survival of infected, thus transmitting, female individuals. Males are a dead end to the bacteria as sperm cells offer too little cytoplasm for them to be transmitted efficiently. Basically, Wolbachia-induced parthenogenesis and cytoplasmic incompatibility may give rise to reproductive isolation of host subpopulations [9,15,16]. Thus, Wolbachia play an important role in the evolution and speciation of the latter [17]. Moreover, the interesting range of effects they may exert in their hosts inspires promising applications in biological control of agricultural pests ([18,19] and references cited therein).

Recently, much effort has been focussed on molecular identification and phylogeny of Wolbachia. Based on 16S rDNA data from over 100 hosts, four major Wolbachia lineages have been recognized hitherto. Groups A and B [16,20] comprise over 90% of all known strains and reside within arthropods, while groups C and D [21] are less documented and harbored in Nematoda. Within groups A and B, there seems to be remarkably little differentiation. The phylogenetic tree derived from ftsZ, a cell division gene having a higher overall molecular clock rate, reflects the 16S tree in a fairly consistent manner, especially emphasizing the differences between the main groups [21,22], although lower-level discrimination among A and B strains turns out to be unreliable here as well [22]. New approaches to small-scale wolbachial evolution using either the heat shock protein groE [23] or the outer surface protein wsp [24] may resolve some inconsistencies provided that the available datasets grow large enough to match those of 16S and ftsZ.

We searched for Wolbachia in invertebrate groups not previously screened. One of the most primitive (entognathous) hexapod orders, the Collembola (springtails), was tackled since one may expect some ‘missing links’ to be disclosed between known cases of Nematoda and Arthropoda.

We observed, by means of transmission electron microscopy (TEM), Wolbachia-like cytoplasmic bacteria in Folsomia candida (Hexapoda, Collembola, Isotomidae). They were discovered by Pálevody and referred to as ‘Rickettsia-like’ [25]. We used TEM to assess the infection in a local parthenogenetic F. candida strain, whereupon we sequenced the endosymbiont 16S rRNA gene, which was found to constitute a novel and primitive branch in the wolbachial phylogenetic tree.

2. Materials and methods

2.1. Animals

A parthenogenetic all-female F. candida stock [26] has been maintained in our laboratory for over 3 years. The animals were kept at ambient temperature in moist vials equipped with a plaster bottom and were fed only baker’s yeast (Saccharomyces cerevisiae) suspended in tap water.

2.2. TEM

Adult F. candida were submerged in fixative (2% paraformaldehyde+2% glutaraldehyde in a 0.06-M cacodylate buffer, pH 7.2–7.4) and beheaded. Fixation took place at 4°C for 24 h after which the specimens were rinsed several times in buffer, incubated overnight at 4°C and post-fixed in 1% OsO₄ in distilled water for 1 h. The specimens were dehydrated, stained en bloc with uranyl acetate and lead acetate and embedded in Araldite CY212 epoxy resin following an established protocol [27]. Thin sections (65 nm) were made using the Reichert-Jung Ultracut E microtome, whereupon they were stained, in the Reichert-Jung Ultrostainer, in a saturated uranyl acetate solution in 50% ethanol (30 min at 40°C) and in alkaline lead citrate (5 min at 30°C), respectively. Preparations were viewed with a JEOL 1200 EX-II.

2.3. DNA purification

The animals were primarily surface-sterilized (1 min) in 70% ethanol and rinsed (3 × 5 min) in sterile physiological water (0.85% NaCl). Whole DNA was prepared from 30 adult females using the Puregene® DNA isolation kit (purchased from BIOzym, The Netherlands) designed for animal tissues and Gram-negative bacteria and following accompanying protocols with slight modifications, taking into account the small scale of the preparation and extending the time for cell lysis to 1 h at 65°C. The same
procedure was carried out on a wild-type *Escherichia coli* strain as a positive control. Upon electrophoresis on 1% agarose gels stained with ethidium bromide, the quality and quantity of the DNA samples, actually containing both host and endosymbiont DNA, were assessed.

### 2.4. PCR amplification of endosymbiont 16S rDNA

PCR reactions were performed in duplicate in 50-μl volumes containing 1.5 mM MgCl₂, 40 μM of each dNTP, 1 μl of each primer (200 ng μl⁻¹), 5 μl (*F. candida* samples) or 1 μl (*E. coli*; positive control) template DNA and 1 U AmpliTaq DNA polymerase (Applied Biosystems, CA, USA). The universal bacterial primers 5’-AGAGTTTGATCCTGGCTCAG-3’ (*E. coli* positions 8–27) and 5’-AAGGAGGTGATCCAGCCGCA-3’ (*E. coli* positions 1541–1522) were used to amplify the nearly complete 16S rRNA gene from the *F. candida* endosymbiont as well as *E. coli*. The following program was run in a Perkin-Elmer Gene Amp 9600 PCR System: 5 min at 95°C, followed by 25 amplification cycles (1 min at 94°C, 1 min at 55°C, 2 min at 72°C) and 10 min final extension at 72°C. PCR products were detected on standard agarose gels with ethidium bromide and those of *F. candida* origin were subsequently purified by means of the QIAquick PCR purification kit and protocol (Qiagen, Germany). A final qualitative and quantitative control was done on agarose as described above.

### 2.5. Cycle sequencing and sequence assemblage

Ten-μl cycle sequencing reactions were set up. To 4 μl Big Dye Terminator Ready reaction mix (Applied Biosystems, CA, USA) was added 3 μl purified PCR product and 3 μl sequencing primer (20 ng μl⁻¹). A set of eight internal 16S primers to conserved regions (*E. coli* positions 339–358, 519–536, 1093–1112, their respective reverse strands and 908–926 and 1222–1241) was used. The reaction profile comprised 30 cycles (15 s at 96°C, 1 min at 55°C, 4 min at 60°C), whereupon all samples were precipitated in 25 μl absolute ethanol to which was added 1 μl of 3 M sodium acetate (pH 4.6). The samples were vortexed, placed on ice for 10 min, centrifuged and ethanol was poured off. DNA pellets were washed in 250 μl ethanol 70% before being vacuum-dried for further use.

Partial sequences were determined using a Perkin-Elmer ABI PRISM® 310 Genetic Analyzer and manually assembled into a nearly complete 16S sequence (1456 bp).

### 2.6. Phylogenetic analysis

A GenBank BLAST search [28] reported our *F. candida* bacterial sequence to be most similar to *Wolbachia* species. Representative *Wolbachia* 16S rDNA sequences were retrieved from GenBank, in addition to several ones from reference groups.

The software package GeneCompar 2.0 (Applied Maths, Kortrijk, Belgium) was used to automatically align (with manual corrections) the sequences according to a modified Needleman-Wunsch algorithm [29], whereupon short entries were discarded until a global alignment was reached of approximately 870 homologous bases, the total alignment was 1598 positions long. Trees were constructed by
means of the neighbor-joining algorithm [30]. Two fundamentally different approaches were followed. In a first instance, GeneCompar global alignment homologies with a gap penalty of 100% were applied to infer the tree topology. Secondly, the global alignment was imported into the TREECON 1.3 package, where the dendrogram was inferred by the substitution rate calibration method [31], taking into account insertions and deletions. The tree presented in this paper (including bootstrap analysis [32]) was calculated in TREECON, after three iterations of substitution rate calibration, and rooted by *E. coli*. The GeneCompar tree did not differ significantly.

3. Results

3.1. TEM

Details obtained with TEM of the endosymbionts

Fig. 2. Neighbor-joining tree, based on wolbachial 16S rDNA sequences. Distances (indels taken into account) were calculated using the substitution rate calibration method in TREECON 1.3. Bootstrap values above 55 are displayed and represent the percentage support for a cluster out of 500 replicates. *Wolbachia* strains are characterized by their host species names (in parentheses) and bear GenBank accession numbers. Group designations are according to literature, except for group E (see Section 4 in this paper). CFP: *Anaplasma marginale* strain from a cystic fibrosis patient.
in *F. candida* are shown in Fig. 1a. They are morphologically very similar to the ones described by Palevody in 1972 [25], although in our case, they never reach the length reported by him (up to 2 μm). Instead, they measure from 0.2 μm to 1.4 μm. Some bacteria are curved to almost hairpin-shaped. Their remarkable flexibility and pleomorphism might result from the lack of any TEM-discernible peptidoglycan in their cell wall, which otherwise resembles the outer membrane typical of Gram-negative bacteria (Fig. 1b). The periplasmic space seems rather narrow (5–15 nm) as compared with the one from *E. coli* (15–20 nm) [33] and contains dense patches in some cells (not shown). It is unclear whether these are artefacts or authentic properties, e.g. of degeneration. Each bacterial cell is surrounded by a host-derived vacuolar membrane, with which it is thought to communicate via outer membrane proteins (Fig. 1b). DNA filaments are visible in a rather diffuse network dispersed throughout the cell and interspersed with ribosomes. No special cellular features such as virus inclusions [34] or crystalline material [35] were observed. The bacteria often occur in aggregations and are found mostly in close association with the rough endoplasmic reticulum (Fig. 1a) in the ovaries, fat bodies and interstitial cells.

3.2. Phylogenetic analysis

The 16S rDNA sequence of the *Wolbachia* group E strain from *F. candida* has been submitted to GenBank under accession number AF179630. Although our *F. candida* endosymbiont shares almost 99% 16S rDNA homology with *A. Wolbachiae*, it presents some unique ‘primitive’ signal sites genuine *A. Wolbachiae* no longer have, to wit: a C at position 177 of the GeneCompar global alignment (like cluster C), a C at position 458 and GCTAG at positions 685–689 (like *Ehrlichia canis* and *Coombie ruminantium*, the most proximal ancestors to *Wolbachia*) and a C at position 1364 (like the group *C. Wolbachia* from the nematode *Dirofilaria immitis*).

The wolbachial phylogenetic tree is shown in Fig. 2. Where possible, group designations are according to recently published analyses. The internal distances of the clusters indicated are below 3%. Five *Wolbachia* lineages appear, four of which correspond to the previously characterized A, B, C and D clusters, respectively. *F. candida* is the host to a distinct *Wolbachia* peripheral to the A-B complex, the latter representing clusters A and B collectively.

4. Discussion

4.1. TEM

Although Palevody [25] did not link the *F. candida* endosymbionts to *Wolbachia*, they were certainly not misplaced within the Rickettsiales order on a morphological basis since they are intravacuolar, mostly very small and pleomorphic. Further confirmation comes with the absence of a detectable peptidoglycan layer in the Gram-negative cell wall, possibly accounting for their flexibility and the utter narrowness of the periplasmic space (5–15 nm). Additionally, the transmission mode was shown to be vertical [25]. The cell size reported was perhaps rather large for *Wolbachia*. In this study, it was more like the one from mosquitos [34,35].

Recently, Braig et al. [36] characterized the gene encoding one of the most abundantly expressed wolbachial proteins: *wsp*, an outer surface protein. The electron-dense particles connecting the outer membrane with the host vacuole (Fig. 1b) might be the best visualization of complexes incorporating these proteins. On these spots, the bacterial outer membrane and the vacuolar membrane are tightly apposed while the periplasmic space remains regular, indicating these complexes may anchor the bacterial outer surface to the host cytoplasmic contents.

4.2. Phylogenetic analysis

The sister groups C and D may be considered the oldest in existence and they seem to have undergone quite some evolutionary changes (as evidenced by their relatively long branches) within their nematode hosts apparently without repeatedly infecting other invertebrates horizontally (= contagious transmission). It is striking that carrier nematodes all belong to the filarias within the evolutionarily recent Spirurida order [37]. This is in agreement with the apparent co-speciation of endosymbiont and host (wolbachial and nematode phylogenies are 100%
congruent!). Furthermore, none of the other orders, including related parasitic nematode orders, have been found to harbor *Wolbachia*.

Clusters E and A-B seem to be poorly bootstrap-supported, perhaps justifying them being considered as E-A-B complex, all the more since the average internal E-A-B distance is only 1.64%. Although group E *Wolbachia* are barely 1.2% distant from cluster A, they should be assigned a separate phylogenetic lineage based on the unique primitive signals (see Section 3) in their 16S rDNA. Two mutually exclusive hypotheses may thus explain the origin of the *F. candida* infection. In the first, a springtail like *F. candida* might have been among the earliest arthropods to have gained a novel *Wolbachia* infection. That this would have happened through contagion by a parasitic carrier nematode seems very unlikely from the dendrogram, in spite of the unique C signal sites in its 16S rDNA. Moreover, filariasis in Collembola has been undocumented hitherto. Furthermore, collembolans are among the most primitive hexapod orders and are peripheral to all insect hosts from the A-B complex. Therefore, *Wolbachia* may have entered the insect world for the very first time in a primitive member from where it was not transmitted horizontally until the far-evolved holometabolic orders (Lepidoptera, Hymenoptera, Coleoptera, Diptera) comprising hosts of cluster A had arisen. Alternatively, the origin of the *F. candida* infection might be found in an as yet undiscovered hexapod host more closely related to those of the A-B complex. One of this hypothetical host’s ancestors may have infected Collembola horizontally. Several routes of horizontal transfer have been discussed [38] and there is evidence of cross-infection in predator-prey [39] and host-parasitoid systems [40].

Various analyses [41,20,21] enlightened the host-endosymbiont co-speciation events in some clades, e.g. among the B Trichogramma species (Hexapoda, Hymenoptera, Trichogrammatidae), the isopods, the C and D *Wolbachia* hosts, as well as the lack of congruence between host and *Wolbachia* evolution in others, the latter being ascribed to horizontal transmission between divergent arthropod orders and best exemplified by the isopod and acarine hosts, which actually branch off quite early in evolution, well before the insects and unlike their respective wolbachiae (Fig. 2).

Coming back to the roots of *Wolbachia*, we conclude that an ‘initial’ *Wolbachia* invasion took place at least twice in the course of evolution: once in an ancestral filarial nematode and once in an ancestral hexapod, the latter being either more primitive (e.g. a collembolan) or more evolved (e.g. related to one of the insect orders harboring A wolbachiae). The subsequent radiation of *Wolbachia* would then result from co-speciation and horizontal transmission events. Alternatively, each *Wolbachia* group may represent an initial invasion whereupon vertical and horizontal transmission ensued to yield the present-day assemblages. As more Collembola and, especially, other primitive insect orders will be investigated, the picture will hopefully become clearer. It remains unclear how many other animal phyla may have been invaded. Finally, it seems acceptable to (i) assign a novel taxon, E, to wolbachiae from the springtail *F. candida* and (ii) maintain a three-species split-up of *W. pipientis*: C, D and the E-A-B complex, although the latter exhibits a certain degree of speciation.

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