Wolbachia-Induced Cytoplasmic Incompatibility to Control Insect Pests?

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ABSTRACT *Wolbachia* are a group of obligatory intracellular and maternally inherited bacteria of arthropods and nematodes, which have recently attracted attention for their potential as new biological control agents. *Wolbachia* are able to invade and maintain themselves in an enormous range of invertebrate species, including insects, mites, spiders, springtails, crustaceans and nematodes. Recent surveys using the polymerase chain reaction (PCR) suggest that perhaps over 20% of arthropod species may be *Wolbachia*-infected, making this bacterium the most ubiquitous intracellular symbiont yet described. *Wolbachia* can manipulate host reproduction by using several strategies, one of which is cytoplasmic incompatibility. *Wolbachia*-induced cytoplasmic incompatibility can be used in several ways: (1) to directly suppress natural arthropod populations of economic and public health importance, (2) as a tool to spread genetically modified strains into wild arthropod populations, and (3) as an expression vector, once a genetic transformation system for this bacterium is developed. A major research aim is to introduce *Wolbachia* into pest and vector species of economic and public health relevance and, through *Wolbachia*-induced cytoplasmic incompatibility, to suppress or modify natural populations.

KEY WORDS *Wolbachia*, cytoplasmic incompatibility, *Ceratitis capitata*, insect pests, biological control

1. Introduction

Wolbachia pipientis Hertig (denoted *Wolbachia* hereafter), is an obligate intracellular and maternally-transmitted bacterium (Werren 1997, Bourtzis and O'Neill 1998, Stouthamer et al. 1999, Stevens et al. 2001, Bourtzis and Miller 2003). *Wolbachia* are able to establish infections in the soma, but they mainly reside in the reproductive tissues of their invertebrate hosts (Fig. 1). *Wolbachia* cause a number of reproductive alterations such as parthenogenetic development, overriding of chromosomal sex determination to convert genetic males into functional females, killing male embryos at early developmental stages, and cytoplasmic incompatibility. Cytoplasmic incompatibility results in mortality of the embryos produced when uninfected females are mated to infected males or when females and males carry incompatible *Wolbachia* strains. Each of these reproductive alterations favours the transmission of the bacterium at the expense of the uninfected arthropod population. Elimination of *Wolbachia* through treatment of the infected hosts with the antibiotic tetracycline results in the restoration of normal reproductive phenotypes.

Wolbachia was first described by Hertig and Wolbach in the 1920s and 1930s as a microorganism infecting the ovaries of mosquitoes belonging to the *Culex pipiens* L. complex, hence the name *W. pipientis* (Hertig 1936). Polymerase chain reaction (PCR) surveys of arthropods, including insects, isopods and mites have indicated the abundance of *Wolbachia* in these organisms (Werren 1997, Werren and O'Neill 1997, Bourtzis and O'Neill 1998, Stouthamer et al. 1999, Stevens et al. 2001, Bourtzis and Miller 2003).

Sequence analysis of the 16S rRNA gene

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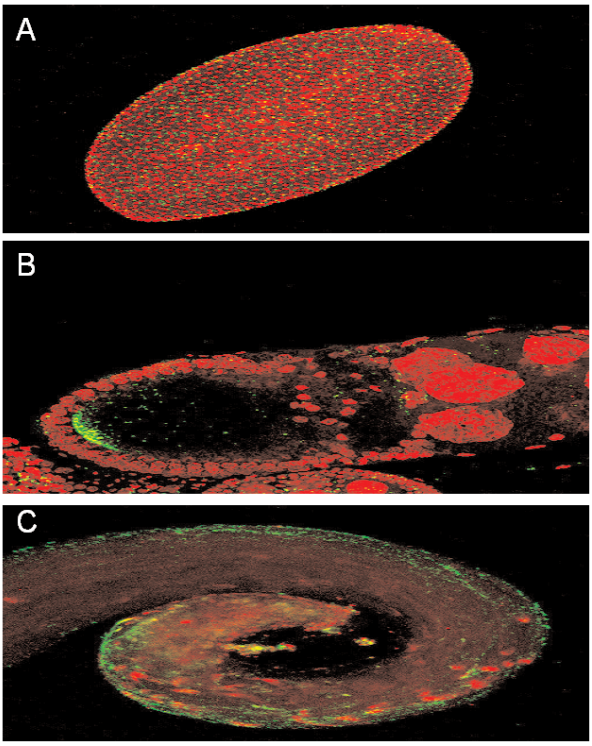

Figure 1. *Presence of* Wolbachia *in infected* Drosophila melanogaster, *(a) embryo, (b) ovary and (c) testes. Bacteria are visualized green-yellow and* Drosophila *nuclei red (Photos by Zoe Veneti and Kostas Bourtzis).*

has shown that *Wolbachia* belong to the alpha-2 subdivision of the Proteobacteria, forming a monophyletic group closely related to intracellular bacteria of the genera Anaplasma, Cowdria, Ehrlichia and Rickettsia (Breeuwer et al. 1992, O'Neill et al. 1992, Rousset et al. 1992). Many members of these genera are arthropod-borne pathogens of mammals. The phylogenies of *Wolbachia* so far generated have shown the existence of six major clades (A-F), which have been named "supergroups" (Lo et al. 2002, and references therein). Supergroups A and B include most of the parasitic *Wolbachia* found to date in arthropods. Supergroups C and D include the majority of the *Wolbachia* found in filarial nematodes. The E supergroup encompasses *Wolbachia* from primitive wing-less insects, the springtails (Collembola). Supergroup F is so far known to infect termites and the filarial parasite *Mansonella ozzardi* (Manson) (Casiraghi et al. 2001). More recently the existence of a new supergroup, named G, encompassing *Wolbachia* from some Australian spiders has been proposed (Rowley et al. 2004).

The mechanism(s) through which *Wolbachia* infects a new species in nature is not yet known. However, *Wolbachia* with a feminizing effect have been successfully transferred by simple haemolymph contact between closely related terrestrial isopod species. This suggests a natural route for inter-individual transfers (Rigaud and Jucqault 1995, Bouchon et al. 1998). Successful *Wolbachia* transfers between different insect species have also been performed. Micro-injection experiments were used to transfer cytoplasmic- and parthenogenesis-inducing

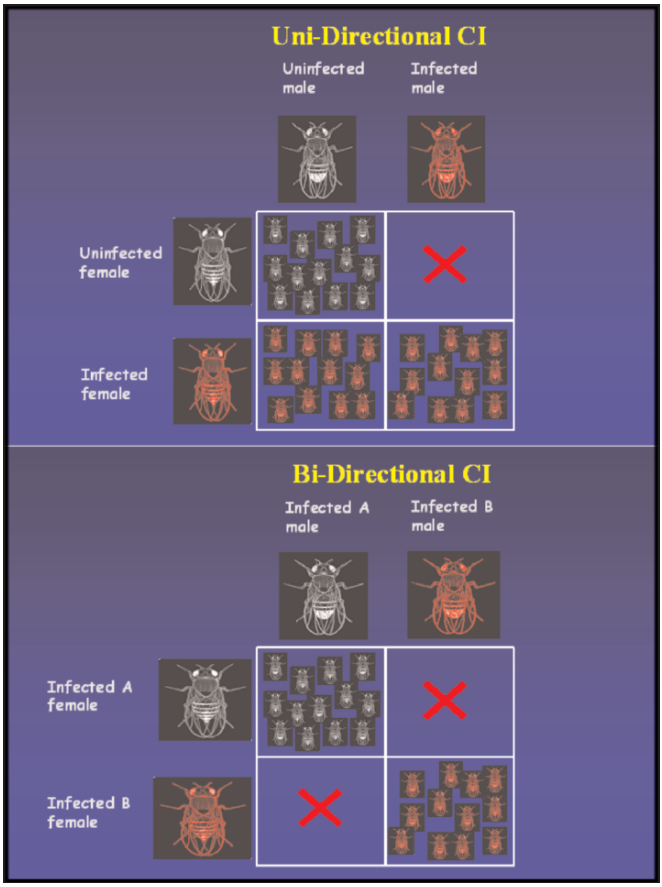

Figure 2. *Schematic representation of cytoplasmic incompatibility, (upper) unidirectional cytoplasmic incompatibility, and (lower) bidirectional cytoplasmic incompatibility (Drawing by Zoe Veneti and Kostas Bourtzis).*

Wolbachia strains between closely and distantly related species. Successful transinfection was followed by the expression of bacterial-induced reproductive phenotypes in the new hosts (Boyle et al. 1993, Braig et al. 1994, Chang and Wade 1994, Rousset and de Stordeur 1994, Giordano et al. 1995, Clancy and Hoffmann 1997, Grenier et al. 1998, Poinsot et al. 1998, Rousset et al. 1999, Riegler et al. 2004, Zabalou et al. 2004a,b). Very closely related *Wolbachia* strains have been found to infect some parasitoid wasps and the insects that they parasitize, which suggests another potential route for horizontal transfer (Werren et al. 1995). In addition, Huigens et al. (2000) reported evidence for horizontal transfer of parthenogenesis-inducing *Wolbachia* under natural conditions. When infected and originally uninfected *Trichogramma kaykai* (Perkins) larvae share a host egg, approximately 40% of the female offspring of the uninfected line acquire the infection and produce some daughters from unfertilized eggs. In subsequent generations, complete (100%) transmission of, and parthenogenesis induction by, *Wolbachia* was observed.

Despite the widespread distribution of *Wolbachia*, many important agricultural pests (e.g. *Ceratitis capitata* (Wiedemann) and

Bactrocera oleae (Gmelin)) and disease vectors (e.g. *Aedes aegypti* (L.) and *Anopheles gambiae* Giles) are not infected.

In this paper, the possible use of cytoplasmic incompatibility as a means for the biological control of insect pests is described, emphasizing the mechanism of *Wolbachia*-induced incompatibility.

2. *Wolbachia*-Induced Cytoplasmic Incompatibility

The phenomenon of cytoplasmic incompatibility was associated with the presence of *Wolbachia* in the 1970s (Yen and Barr 1971, 1973). Cytoplasmic incompatibility results in embryonic mortality in crosses between insects with different *Wolbachia* infection status. It can be either uni- or bidirectional (Fig. 2). Unidirectional cytoplasmic incompatibility is typically expressed when an infected male is crossed with an uninfected female. The reciprocal cross (infected female and uninfected male) is fully compatible, as are crosses between infected individuals. Bidirectional cytoplasmic incompatibility occurs in crosses between infected individuals harbouring different strains of *Wolbachia* strains, that is, strains with different modification and rescue properties. In most insects, the expression of cytoplasmic incompatibility is lethal to the developing embryo. In insects with haplodiploid sex determination (Hymenoptera), the result of cytoplasmic incompatibility is a sex ratio shift to the haploid sex, which is usually male.

Cytoplasmic incompatibility has been documented in diverse insect taxa including Coleoptera, Diptera, Hemiptera, Orthoptera, Hymenoptera, and Lepidoptera, as well as in the terrestrial isopod *Porcellio dilatatus* Brandt & Ratzeburg and in mites (Werren and O'Neill 1997). The host nuclear genome, the age of the male, repeated copulation of males and several environmental factors such as temperature, antibiotics, nutrition and larval density greatly influence the strength of the cytoplasmic incompatibility phenotype (Bourtzis et al. 2003). A correlation between *Wolbachia* density and the level of incompatibility has been demonstrated in several systems (Bourtzis et al. 2003).

The mechanism(s) by which *Wolbachia* causes cytoplasmic incompatibility have not yet been identified. However, a number of cytogenetic studies have described the events that take place during and shortly after fertilization in incompatible crosses. These studies described developmental defects and aberrant DNA structures in incompatible crosses as early as the first mitotic division, demonstrated that the paternal chromosome decondensation is delayed leading to improper paternal chromatin inheritance and to the production of embryos with aneuploid or haploid nuclei, and allowed observation of the direct interaction between *Wolbachia* and astral microtubules (Jost 1970, O'Neill and Karr 1990, Callaini et al. 1994, Kose and Karr 1995, Lassy and Karr 1996, Callaini et al. 1996, 1997). In a recent and very elegant study in Nasonia, Tram and Sullivan (2002) used real-time imaging and indirect immunofluorescence to visualize early developmental events leading to the expression of cytoplasmic incompatibility and consequent egg lethality and concluded that *Wolbachia* affects the timing of nuclear envelope breakdown prior to the crucial first gonomeric division. These and previous results showed convincingly that *Wolbachia* somehow modifies the paternal chromosomes during spermatogenesis (mature sperm do not contain the bacteria) thus influencing their fate during the first mitotic divisions and resulting in loss of mitotic synchrony.

Based on the genetic and cytogenetic data, Werren (1997) proposed the so-called modification/rescue model, which assumes the presence of two distinct bacterial functions. First, the modification function, a kind of "imprinting" effect, which acts in the male germ-line, probably during spermatogenesis, and second, the rescue function, which acts in the egg. Sperm imprinting may be due either to secreted *Wolbachia* protein(s) that modify the paternal chromosomes or to the removal of host protein(s) that are necessary for proper condensation/decondensation of the paternal chro-

mosomal set before and/or during zygote formation. Similarly, the presence of the same *Wolbachia* strain in the egg may result in the production and secretion of (a) rescue factor(s), or alternatively the recruitment of host molecules which are capable of rescuing the sperm "imprint" in a *Wolbachia* strain-specific manner.

Recently the genome sequence of two *Wolbachia* strains was reported: the first, *w*Mel, belongs to the *Wolbachia* strain infecting *Drosophila melanogaster* Meigen (Wu et al. 2004); the second, *w*Bm, belongs to the *Wolbachia* strain infecting the filarial nematode *Brugia malayi* (Brug) (Foster et al. 2005). The available genomic information provides the necessary tools to undertake comparative post-genomics approaches towards the identification of the genes involved in *Wolbachia*-host interactions thus deciphering the biology of this unculturable bacterium including the mechanism of cytoplasmic incompatibility, understanding *Wolbachia*-host symbiotic associations and uncovering the evolution of intracellular symbiosis.

3. Cytoplasmic Incompatibility-Inducing *Wolbachia* and Applications

Wolbachia has been suggested as a potential tool for the development of novel, environment-friendly strategies for the control of arthropod species that are major agricultural pests or disease vectors to humans, plants, and livestock or for improving beneficial species (Beard et al. 1993, Bourtzis and O'Neill 1998, Bourtzis and Braig 1999). Below is an outline of the potential applications for cytoplasmic incompatibility-inducing strains of *Wolbachia*.

3.1. Release of Infected Sterile Males

Wolbachia-induced cytoplasmic incompatibility might be used to suppress natural populations of arthropod pests in a way analogous to the sterile insect technique (SIT). The SIT involves mass-production and release of irradiated sterile insects and is one of the methods used within area-wide integrated pest management (AW-IPM) programmes for the control of insect pests (Dyck et al. 2005). Cytoplasmic incompatibility provides an alternative method to produce non-irradiated sterile males that may have improved competitiveness and so improve the efficiency of the SIT.

Recently the transfer of *Wolbachia* strains from the European cherry fruit fly *Rhagoletis cerasi* L. (Riegler and Stauffer 2002) led to stable infections in the Mediterranean fruit fly, following embryonic injection (Zabalou et al. 2004a). Austrian and Sicilian populations of *R. cerasi* were used as donors carrying different combinations of four *Wolbachia* variants (Riegler and Stauffer 2002, Zabalou et al. 2004a, M. Riegler and C. Stauffer, unpublished, cited in Zabalou et al. 2004a). Two out of initially eleven positive transinfected isofemale lines remained positive for the presence of *Wolbachia*, namely WolMed 88.6 (single infection with *w*Cer2) and WolMed S10.3 (single infection with *w*Cer4). At the time of writing, 47 generations (about 41 months) post-infection, both lines are stably infected with infection rates of 100%.

Test crosses were performed in different generations post-injection between transinfected lines and the parental uninfected Mediterranean fruit fly strains. All crossing experiments showed the same results: crosses between uninfected females and *Wolbachia*-infected males resulted in 100% egg mortality. Similar results were obtained in test crosses performed three years post-injection (unpublished). It has to be noted that complete cytoplasmic incompatibility has only been observed in very few *Wolbachia*-infected species such as *C. pipiens* (Laven 1967). This was the first report that a newly transinfected host species shows high stability of the infection and, at the same time, expresses 100% cytoplasmic incompatibility (unidirectional and bidirectional).

Laboratory cage populations of Mediterranean fruit flies containing different ratios of transinfected males:uninfected males:uninfected females were set up to deter-

mine whether cytoplasmic incompatibility expressed by the *Wolbachia*-infected lines could be used for population suppression. The caged Mediterranean fruit fly populations were suppressed by these single "releases" of incompatible males in a ratio-dependent manner. Population suppression was extremely efficient reaching levels greater than 99% at transinfected males:uninfected male release ratios of 50:1. Although these laboratory experiments are very encouraging they need to be extended to field cage systems where wild flies are used as the target population.

For effective *Wolbachia*-based population suppression, an efficient (100% effective) genetic sexing system producing only males is necessary and there are intensive efforts ongoing using both genetic and molecular approaches to develop such sexing systems in a variety of pest species. However as yet, none of the systems so far tested would meet the requirements needed in order to exclude the last female from the release males. Given this requirement and the numbers of insects that need to be released it is unlikely that an operational fail-safe sexing system can be developed. However using a sexing system such as that currently being used for the Mediterranean fruit fly (Franz 2005), several strategies could be considered. One solution may be to have two bidirectionally incompatible infected strains where males from the two strains are released alternately, so that even if an infected female of the one strain is released then in the next generation her offspring will most likely mate with males infected with the incompatible sperm. An alternative solution may be to combine radiation with incompatibility where the contaminating females can be sterilized with lower doses of radiation than males. In this way the released males could be more competitive as they will receive a lower dose of radiation. In tephritid species, females are sterilized by lower doses of radiation than males (Bakri et al. 2005).

Cytoplasmic incompatibility has been used in the past to introduce sterility into wild populations of mosquitoes. Indeed, several trials sponsored by the World Health Organization, were undertaken in the mid 1960s in Burma and India to eradicate the filariasis vector species *C. pipiens* and *Culex quinquefasciatus* Say. By mass-rearing and then releasing males that were incompatible with the target population, it was possible to effectively sterilize wild females. In one field trial, mosquitoes were completely eradicated from a Burmese village (Laven 1967). Also, in the 1970s, an international collaborative project took place in Central Europe, which evaluated cytoplasmic incompatibility as a method to control the European cherry fruit fly *R. cerasi*. Several successful field trials were performed, but for a number of reasons, this project was never completed (Blümel and Russ 1989, Boller 1989). In addition to these field experiments, a number of laboratory and warehouse experiments in the USA have successfully applied *Wolbachia*-induced cytoplasmic incompatibility as a means to control the stored product pest, the almond moth *Cadra* (*Ephestia*) *cautella* Walker (Brower 1978, 1979, 1980).

3.2. Release of Infected Fertile Male and Female Insects to Spread Wolbachia *in a Natural Pest Population*

Wolbachia-induced cytoplasmic incompatibility might be used as a mechanism to spread desirable genotypes into field populations (Turelli and Hoffmann 1991, Hoshizaki and Shimada 1995, Sinkins et al. 1995, Hoshizaki 1997, Rousset et al. 1999). The identification of the *Wolbachia* genes responsible for cytoplasmic incompatibility should allow the introduction of these genes into the host nuclear genome and the induction of cytoplasmic incompatibility without the presence of *Wolbachia*. Theoretical models suggest that nuclear-coded cytoplasmic incompatibility genes will lead to a spread of their host, replacing target naïve populations along with any other chromosomally-linked gene(s) (Sinkins et al. 1997, Curtis and Sinkins 1998, Sinkins and Godfray 2004).

Recently, Xi and colleagues reported the transfer of a *w*AlbB *Wolbachia* strain naturally occurring in *Aedes albopictus* Skuse, and

its establishment in *Ae. aegypti*, a naïve host (Xi et al. 2005). Crossing experiments indicated strong cytoplasmic incompatibility (100%): no egg hatch observed from more than 3800 eggs examined from crosses of uninfected females and *Wolbachia*-infected males. Laboratory cage tests demonstrated that *Wolbachia* can be spread into a targeted uninfected *Ae. aegypti* population, reaching infection fixation within seven generations. This is the second report that a newly transinfected host species shows high stability of the infection and, at the same time, expresses 100% cytoplasmic incompatibility. In addition, these data clearly indicated that *Wolbachia* can be used as a vehicle to drive transgenes into mosquito populations, and maybe other disease vector populations of medical importance.

3.3. Release of Male and Female Insects to Spread Paratransgenic Wolbachia

Wolbachia might be also used as an expression vector in paratransgenesis strategies. Paratransgenesis is a method that uses symbiotic bacteria as vehicles for the introduction and expression of genes of interest into a target arthropod species and has been suggested as an alternative approach for the genetic manipulation of arthropods (Beard et al. 1993, Ashburner et al. 1998). Symbiotic bacteria of arthopod species have already been used as expression vehicles (Durvasula et al. 1997, Cheng and Aksoy 1999) and a paratransformation approach is currently being evaluated for field releases of *Rhodnius prolixus* (Stål) aiming to reduce the prevalence of the causative agent of Chagas' disease *Trypanosoma cruzi* Chagas (Durvasula et al. 1997). The main obstacle to using *Wolbachia* in paratransgenesis approaches is that it cannot be cultured in a cell-free system, and a genetic transformation system is not yet available. The fact that these bacteria can now be maintained in different insect cell lines (O'Neill et al. 1997, Dobson et al. 2002) coupled with the recent isolation and characterization of endogenous phages and insertion sequences (Masui et al. 2000, 2001, Fujii et al. 2004), will certainly facilitate current efforts to genetically engineer *Wolbachia*.

4. Conclusions

Wolbachia-based applications may be broad since these bacteria are present in a wide range of arthropod species and can also be transferred into naïve hosts. It is possible that the ability of these bacteria to establish new infections and persist in their hosts for a long time may have to do with their ability to "escape" the host's innate immune system (Bourtzis et al. 2000). However, and despite the potential demonstrated in the above-mentioned earlier trials, there has been no consistent experimental follow-up with the exception of several review papers (Sinkins et al. 1997, Bourtzis and Braig 1999, Sinkins and O'Neill, 2000, Aksoy et al. 2001, Bourtzis and Robinson 2006). Therefore it remains to be demonstrated whether *Wolbachia*-based technologies will be used in the field and ever replace and/or complement existing operational AW-IPM programmes.

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