RESEARCH LETTER

A novel approach to eliminate *Wolbachia* infections in *Nasonia vitripennis* revealed different antibiotic resistance between two bacterial strains

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Abstract

Wolbachia are widespread in insects and can manipulate host reproduction. *Nasonia vitripennis* is a widely studied organism with a very high prevalence of *Wolbachia* infection. To study the effect of *Wolbachia* infection in *Nasonia* spp., it is important to obtain noninfected individuals by artificial methods. Current methods that employ sugar water-containing antibiotics can successfully eliminate *Wolbachia* from the parasitic wasps; however, treatment of at least three generations is required. Here, we describe a novel, feasible, and effective approach to eliminate *Wolbachia* from *N. vitripennis* by feeding fly pupae continuously offering antibiotics to *Nasonia* populations, which shortened the time to eliminate the pathogens to two generations. Additionally, the *Wolbachia Uni* and *CauB* strains have obviously different rifampicin-resistance abilities, which is a previously unknown phenomenon.

Introduction

Wolbachia is a genus of intracellular maternally inherited α-proteobacteria prevalent in the majority of arthropods and filarial nematodes. Moreover, the major vectors of these bacteria are insects (Baldo *et al.*, 2008; Serbus *et al.*, 2008). *Wolbachia* infection is associated with a variety of reproductive anomalies in the hosts, including cytoplasmic incompatibility, parthenogenesis, male lethal, and feminization (Duron *et al.*, 2008). Many studies have been devoted to the effects of this fascinating bacterial group.

Nasonia (Order *Hymenoptera*; Family *Pteromalidae*) is a complex of four parasitic wasp species that has emerged as a model for evolutionary and developmental genetics (Werren & Loehlin, 2009a, b). *Nasonia* species are haplodiploid, in which haploid males develop from unfertilized eggs and diploid females develop from fertilized eggs (Pultz & Leaf, 2003; Beukeboom *et al.*, 2007). The adult females deposit fertilized and unfertilized eggs inside the pupal cases of larger dipterans, such as *Musca domestica* (Pultz & Leaf, 2003). Furthermore, *Nasonia* species are reportedly often infected with *Wolbachia* (Bordenstein & Werren, 1998; Bordenstein *et al.*, 2003; Raychoudhury *et al.*, 2009), which makes them a good model to study the effect of *Wolbachia* in insects. *Wolbachia* of the supergroups A and B commonly infect *Nasonia vitripennis* (Werren *et al.*, 1995; Bordenstein *et al.*, 2001), resulting in cytoplasmic incompatibility (Tram *et al.*, 2006; Clark *et al.*, 2008). The common presence of double or single infections with two distinct bacterial strains (A and B) in *N. vitripennis* have been confirmed by both polymerase chain reaction (PCR) amplification and Southern blot analysis of genomic DNA (Perrot-Minnot *et al.*, 1996).

Wolbachia are primarily transmitted vertically from mother to offspring via infected eggs and less often horizontally (Hughes *et al.*, 2004; Kassem & Osman, 2007; Narita *et al.*, 2009). This complex pattern of transmission results in a high prevalence of *Wolbachia* infection of *N. vitripennis*; thus, it is difficult to find uninfected individuals in nature. To reveal the biological significance of *Wolbachia* in this model insect, it is crucial to obtain a sufficient number of uninfected individuals by artificial methods. Previous approaches to cure *Wolbachia* infections in adult wasps by feeding the insects sugar water supplemented with antibiotics have worked well (Kyei-poku *et al.*, 2003; Werren & Loehlin, 2009a, b). However, this

procedure requires treatment of at least three consecutive generations (each generation can take 15 days) up to six generations to completely eliminate *Wolbachia*. Thus, it takes a relatively long time to re-establish an uninfected strain using conventional approaches. In the present study, we developed a new approach to establish uninfected wasps by providing the insects with antibiotic-treated housefly pupae in replacement of sugar water-containing antibiotics, resulting in a decrease in the time needed for *Wolbachia* elimination to two generations. We also compared rifampicin resistance between two divergent *Wolbachia* strains in *N. vitripennis*, which have distinct divergent antibiotic resistance.

Materials and methods

Species studied and sample collection

A clonal population of *M. domestica* L. (*Diptera*; *Muscidae*) and two clonal populations of *N. vitripennis* (Walker) (*Hymenoptera*; *Pteromalidae*) (each established from a single parthenogenetic female) were used in the experiments. The founder *N. vitripennis* populations were collected from different geographically isolated field populations in Anhui Province and Zhejiang Province, China. The housefly larvae were fed bran for 6 days until pupation. All wasps were reared on fresh house fly pupae at 25 ± 2 °C under a 14-h light cycle in an atmosphere of 50–60% relative humidity, supplemented with a piece of cotton in a soft capsule shell of 10% honey water. The adult houseflies were kept under the same conditions, but supplied with a sugar/milk powder mixture (25% : 75%) and water instead. Randomly selected adults of *M. domestica* and *N. vitripennis* were stored in 95% EtOH at −20 °C until further testing of *Wolbachia* infection.

Antibiotic treatments

We provided wasps with antibiotic-treated housefly pupae instead of sugar water-containing antibiotics. Rifampicin was combined with bran as a food source for *M. domestica* larvae (at dry weight doses of 0% (control), 0.3%, 0.45%, 0.7%, 0.9%, and 1.3% respectively), in which housefly eggs were located until pupation. The antibiotic-treated pupae were then provided to adult female wasps to deposit fertilized and unfertilized eggs. An untreated group was established as a control.

DNA extraction

DNA was extracted from 4-day-old adult wasps and houseflies. Insects were immersed for 5 min in a 70% ethanol solution, washed for 5 min in sterile distilled water, and crushed individually in sterile Eppendorf tubes with the tip of a sterile pipette. DNA was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA extraction was performed simultaneously for all individuals to ensure uniformity and the fresh weight of each individual was measured before DNA extraction.

Detection of *Wolbachia*

Seven groups of samples are listed in Table 1, including (1 and 2) *N. vitripennis* controls (no antibiotic) from both the Zhejiang and Anhui populations; (3 and 4) *N. vitripennis* from each population treated once with 0.7% rifampicin; (5 and 6) *N. vitripennis* from each population treated twice in succession with 0.7% rifampicin; and (7) *M. domestica* without antibiotic treatment. For each group, 10 male and 10 female wasps reared from several host flies were randomly chosen to detect the *Wolbachia* infection pattern by PCR amplification with three pairs of *Wolbachia*-specific primers. Primers for the mitochondrial cytochrome oxidase I (COI) gene were used to test the quality of extracted DNA. To ensure the reliability of the data, we demonstrated altogether three rounds of independent experiments on independently produced groups of rifampicin-treated flies. The same treated host flies were used for both populations of

Table 1. The efficacy of rifampicin on *Wolbachia* infections in *Nasonia vitripennis*

Species	*Wolbachia* strain	Antibiotic treatment	Average of infection rate (based on 10♂ and 10♀ samples for each group)	Others
N. vitripennis (Zhejiang)	*Uni* (A)	None Once Twice (successive)	100% 83.3% 0	Individual males and females of lines Zhejiang and Anhui (without antibiotic treatment) were also used in measuring bacterial density
N. vitripennis (Anhui)	*CauB* (B)	None Once Twice (successive)	100% 0 0	
M. domestica	None	None	0	N/A

wasps. All primers used in the experimentation are listed in Supporting Information, Table S1.

A total volume of 2.5 μL of extracted DNA was amplified in a 25-μL reaction mixture containing 12.5 pmol of each primer, 200 μM of dATP, dCTP, dGTP, and dTTP, and 1 U of Elongase in 1X PCR buffer with 0.8 μL of 25 mM $MgCl_2$ (Life Technologies, Shanghai, China) (Rolain *et al.*, 2003). All PCRs were performed using a Peltier Thermal Cycler PTC-200 (MJ Research, Inc., Watertown, MA) with an initial 5-min denaturation step at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C (16S rRNA gene), 55 °C (*COI* and *ftsZ*), or 59 °C (*wsp*) for 40 s, and extension at 72 °C for 1 min. Amplification was completed by continuing the reaction at 72 °C for 10 min to allow complete extension of the PCR products. All PCR products were visualized under ultraviolet light after electrophoresis in 1% agarose gel and stained with ethidium bromide. For PCR procedures, negative controls consisted of sterile distilled water added to a sample of PCR mix instead of DNA and the sample of DNA from a fig wasp species, which had a high *Wolbachia* infection titer (H.-Y. Liu & D.-W. Huang, unpublished data), was included in each PCR as a positive control.

Cloning and sequencing

PCR products of the *wsp* gene were purified using QIAquick PCR purification columns (Qiagen Inc., Valencia, CA) and then ligated into pGEM-T vectors (TA cloning kit; Promega Corp., Madison, WI) and sequenced by the BioSun Sequencing Center (Beijing, China).

Sequence data analysis

Partial *wsp* gene sequences were aligned using the ClustalW Multiple Sequence Alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2) and then manually modified based on the amino acid translations. The resulting data set included 517 nucleotide sites and the sequences were aligned with a subset of 28 previously published *Wolbachia* sequences from other insects and arthropods (Zhou *et al.*, 1998) (Table S2). A distance matrix was calculated using the Molecular Evolutionary Genetics Analysis algorithm (version 5.0; http://www.megasoftware.net) with a p-distance metric and pairwise deletion of insertions and deletions. Phylogenetic trees were constructed from this matrix. Bootstrap analysis, using 500 pseudoreplicates, was used to test the reliability of the clades in the phylogeny, as shown in Fig. 1.

Real-time quantitative PCR (RT-qPCR)

Bacterial density was measured in 4-day-old males and females reared under standardized conditions. RT-qPCR was performed using the Light Cycler™ System (Roche Applied Science, Penzberg, Germany). In RT-qPCR, as the number of PCR cycles increases, the amount of amplified PCR product is monitored using a fluorescent-labeled double-stranded DNA binding dye (SYBR Green I Dye; Life Technologies, Carlsbad, CA). The system measures threshold cycles once fluorescence increases from the background level. Standard curves were drawn using the *Wolbachia* strain clone obtained from infected females after amplification with WspQF1/WspQR1 primers and plotted using five dilutions (from 10^{-5} to 10^{-9}) of this vector containing one copy of a specific *wsp* sequence. General primers of *wsp* (WspQF1, 5′-TGCAGACAGTT TAACAGC-3′; WspQR1, 5′-TAAGAACCGAAATAGC GA-3′) were used to amplify a DNA fragment of about 246 bp. The 20-μL reaction mixture contained 10% (v/v) Light Cycler DNA master SYBR Green I (Roche Diagnostics, Meylan, France), 3 mm of $MgCl_2$, 500 nm of each primer, and 2 μL of template DNA. Each RT-qPCR cycle was performed under the following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s and 54 °C for 30 s.

The number of *Wolbachia* bacteria was calculated as described by Noda *et al.* (2001), assuming *wsp* as a single-copy gene (Braig *et al.*, 1998). To obtain the density, the number of bacteria was corrected with the fresh weight of the wasp measured before DNA extraction.

Results and discussion

Wolbachia strains recovered from two *N. vitripennis* populations

The *Wolbachia wsp* sequences were obtained from two laboratory-maintained populations of *N. vitripennis*. A neighbor-joining tree was constructed from these sequences together with other similar *Wolbachia* sequences retrieved from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov; Fig. 1), which showed that the *wsp* sequences of the two populations were also harbored by other strains. *Wolbachia* sequences obtained from the *N. vitripennis* of Zhejiang Province (ZJNW) were placed in the *Uni* group of supergroup A, while those from Anhui Province (AHNW) were placed in the *CauB* group belonging to supergroup B.

We also measured the infection titer of both *Wolbachia* strains in their hosts by real-time PCR (Table 2). The average infection titer of the Zhejiang samples of the *Uni* group was lower than that of Anhui samples in the *CauB* group. Within each population, the titer in females was slightly higher than that in males, which indicated that males were more likely to have lost *Wolbachia* infections than females stochastically.

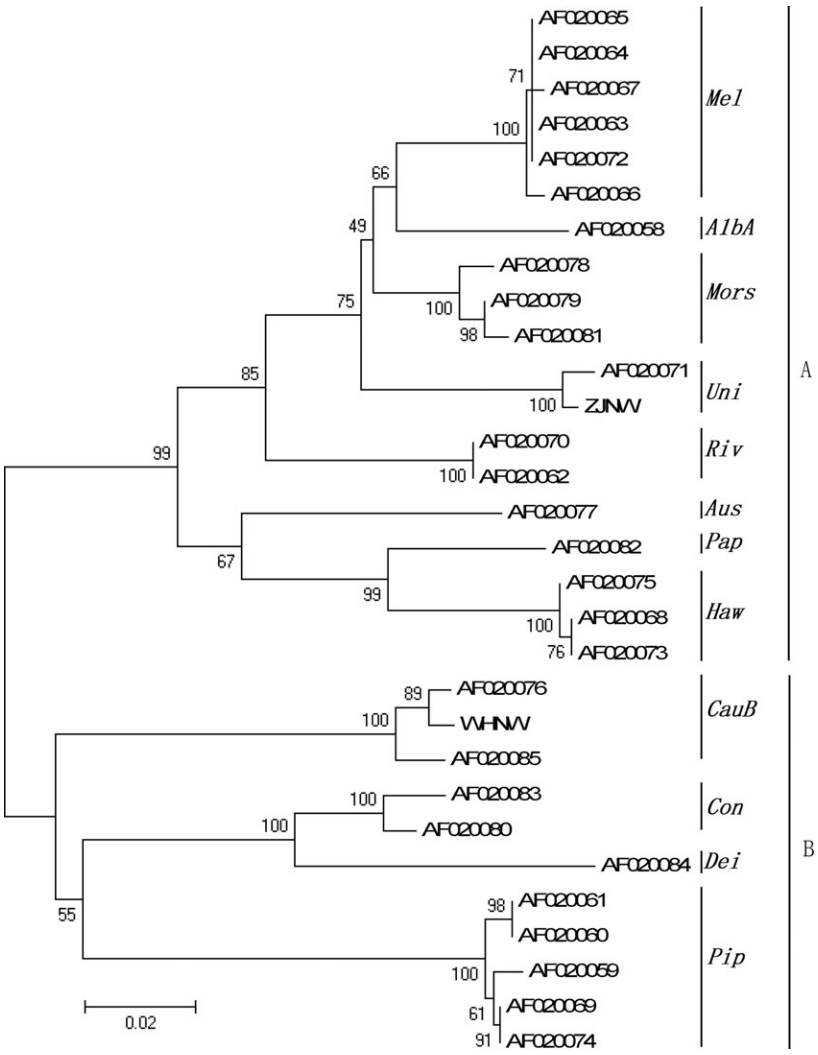

Fig. 1. Neighbor-joining tree constructed with the *wsp* sequences of thirty different *Wolbachia* strains using a ClustalW alignment (the hypervariable regions of the gene were excluded). Taxa are labeled as the numbers representing the host. The information of these numbers is provided in Table S2. ZJNW, *Wolbachia* in *Nasonia vitripennis* of Zhejiang Province; WHNW, *Wolbachia* in *N. vitripennis* of Anhui Province.

Effects of rifampicin on *M. domestica* and *N. vitripennis*

We tested the effects of different rifampicin concentrations in bran on the growth of the *M. domestica* larvae. Antibiotic concentrations lower than 0.7% (dry weight) had no obvious effect on larval development. However, rifampicin concentrations > 0.7% extended the time of pupation, reduced the size of the larvae, and decreased larval activities. As shown in Table 3, rifampicin concentrations ≥ 1.3% were lethal to the larvae.

Based on the above results, we chose an antibiotic concentration of 0.7% for treatment of *M. domestica* larvae. The antibiotic-treated housefly pupae were offered to the infected female wasps (fertilized), so that the wasp larvae would be constantly treated with rifampicin until pupation. Two laboratory-maintained strains of *N. vitripennis* with no apparent differences in life span and body size compared to antibiotic-free control wasps were tested in this experiment.

Efficacy of rifampicin on *Wolbachia* strains

As shown in Table 1, rifampicin efficacy differed between the two strains of *N. vitripennis* infected with different strains of *Wolbachia* (infection prevalence, 100%). For *N. vitripennis* from Zhejiang, which was infected with a *Wolbachia* strain of the *Uni* group, after one antibiotic treatment, the prevalence of *Wolbachia* in the offspring decreased to 83.3% and a successive second round of

Table 2. *Wolbachia* infection titer detected by real-time qPCR

Line	Sex	No. of *Wolbachia* per wasp	Fresh weight per wasp (mg)	No. of *Wolbachia* per mg of wasp (10^6)
Anhui (*CauB*)	Female	2.04×10^6	0.5	4.08
		2.04×10^6	0.5	4.08
		$2.04 \times 10^{5.9}$	0.49	3.31
		$2.04 \times 10^{5.9}$	0.49	3.31
		$2.04 \times 10^{5.9}$	0.48	3.38
	Male	$2.04 \times 10^{5.2}$	0.32	1.01
		2.04×10^5	0.29	0.70
		2.04×10^5	0.29	0.70
		$2.04 \times 10^{4.9}$	0.28	0.58
		$2.04 \times 10^{4.8}$	0.28	0.46
Zhejiang (*Uni*)	Female	$2.04 \times 10^{4.9}$	0.5	0.32
		$2.04 \times 10^{4.8}$	0.49	0.26
		$2.04 \times 10^{4.8}$	0.49	0.26
		$2.04 \times 10^{4.6}$	0.48	0.17
		$2.04 \times 10^{4.6}$	0.47	0.17
	Male	$2.04 \times 10^{4.3}$	0.30	0.14
		$2.04 \times 10^{4.2}$	0.29	0.11
		$2.04 \times 10^{4.2}$	0.29	0.11
		2.04×10^4	0.27	0.08
		2.04×10^4	0.27	0.08

Table 3. The development of the larva of *Musca domestica* after treated with different concentrations of rifampicin

Antibiotics concentration	Larvae's size before pupation	Time needed for pupation	Larvae's activity
0%	Normal	6 days	Active
0.3%	Normal	6 days	Active
0.45%	Normal	6 days	Active
0.7%	Slightly small	7 days	Weakened slightly
0.9%	Small	> 7 days	Weakened
1.3%	Small	Diapause and then dead	Inactive

antibiotic treatment eliminated all of the *Wolbachia* from the offspring. However, rifampicin seemed to have stronger efficacy on the *N. vitripennis* from Anhui Province infected with a *Wolbachia* strain of the *CauB* group, which was totally eliminated after one antibiotic treatment. There was no rejuvenation in the third generation.

Here, we present a novel approach to eliminate *Wolbachia* infections in *N. vitripennis*. Compared to previous approaches, we replaced sugar water-containing antibiotics with antibiotic-treated house fly pupae to more easily deliver constant antibiotic treatment to the wasps. The time needed for total elimination of *Wolbachia* was shortened to only two generations, and there was no rejuvenation in the third generation, which indicated that our approach was effective.

In our experiments, precise treatment of houseflies was a key step for the success of the approach. First, we verified that all of the houseflies used in the experiment were free from *Wolbachia* to avoid re-infection by confirming the absence of the three *Wolbachia* genes *wsp*, 16S rRNA gene, and *ftsZ* by RT-qPCR. Second, we verified that the housefly pupae fed to the wasps received constant antibiotic supplementation with the least negative effect on the development of the houseflies, because normal growth and development of the wasps were dependent on healthy housefly pupae. We made detailed comparisons on the effects of different rifampicin concentrations on the development of houseflies, including body size, activity, and time needed for pupation to choose the most appropriate concentration.

The RT-qPCR results provided a good estimation of the *Wolbachia* titer in both populations, which indicated that the titer of the *CauB* strain in the Anhui population was much higher than that in the *Uni* strain in the Zhejiang population. However, the *CauB* strain was totally eliminated after only one antibiotic treatment, whereas the *Uni* strain was detectable in 83.3% of individuals of the Zhejiang population after one treatment; thus, a second round of antibiotic treatment was needed to completely remove the bacteria. This evidence indicated that

the lower titer of the *Wolbachia Uni* strain had greater rifampicin resistance than the higher titer of the *CauB* strain. The presence of the small proportion of Zhejiang population (16.7%) in which the *Wolbachia* could be eliminated after only one antibiotic treatment may indicate that in some stochastic conditions, for example, when fly hosts carry higher antibiotic titers, the reared wasps may thus load a lower number of *Wolbachia*, which were easier to eliminate by use of the antibiotic. In addition, our extensive experience raising wasps indicated that cooler temperatures impacted survival more severely in the Anhui population compared to the Zhejiang population. The difference in antibiotic resistance and *Wolbachia* load of the *Uni* and *CauB* strains between the different wasp populations may be due to genetic differences in the bacteria or in their wasp host. The role of host effects can be determined by backcrossing the *Wolbachia* into each respective host's nuclear background (Werren & Loehlin, 2009a, b).

In conclusion, *N. vitripennis* wasps have a high prevalence of *Wolbachia* infection and were used to explore the effects of *Wolbachia* on insects. Therefore, it is essential to efficiently obtain a sufficient number of uninfected individuals. Here, we described a new approach to establish *N. vitripennis* without *Wolbachia* infection using antibiotic-treated house fly pupae, which is much easier and effective. We also compared rifampicin resistance between two divergent *Wolbachia* strains in *N. vitripennis* and found distinct differences. Therefore, future research is warranted to determine whether different *Wolbachia* strains have different effects in the same host.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers for amplifications in this study.
Table S2. *Wolbachia* group nomenclature.