Wolbachia are intracellular bacteria present in a wide range of arthropods, particularly in insects. In a previous field survey by Werren et al. (1995b), Wolbachia were found in more than 16% of the insects sampled. Phylogenetic analyses of the Wolbachia harboured by various host species have shown that the Wolbachia clade can be divided into two major groups, designated A and B (Werren et al., 1995a).

Wolbachia are known to induce various reproductive alterations in their hosts, including cytoplasmic incompatibility, thelytokous parthenogenesis, feminization and male-killing. In this study, we examined Wolbachia infection and its effects on the host cricket Teleogryllus tawanemma. In a phylogenetic study based on the wsp gene coding for a Wolbachia surface protein, the Wolbachia strain harboured by T. tawanemma was clustered together with those harbourered by Laodelphax striatellus, Tribolium confusum, Acraea encedon, Trichogramma deion and Adalia bipunctata. Crossing experiments using the Wolbachia-infected and uninfected strains of cricket showed that the infection is associated with the expression of unidirectional cytoplasmic incompatibility: the egg hatch rate in the incompatible cross between the infected males and uninfected females was 20.3%. We also examined the distribution of Wolbachia within the host using polymerase chain reaction assays; they were detected in the antennae, heads, forewings, hindwings, testes, ovaries, Malpighian tubules, foot muscles and fat bodies. Quantitative polymerase chain reaction assays showed that the bacterial density was highest in the fat bodies, followed by the ovaries and testes. Wolbachia were not detected in the haemolymph or in mature spermatoozoa. The spermatozoa of the infected male may be modified by the presence of Wolbachia during its development. To examine this possibility, we compared the profiles of sperm proteins between the infected and uninfected males using two-dimensional gel electrophoresis. However, no differences in the protein profiles were observed.

Key words: Wolbachia, cricket, Teleogryllus tawanemma, cytoplasmic incompatibility, bacterial density, two-dimensional gel electrophoresis.

Introduction

Wolbachia are intracellular bacteria present in a wide range of arthropods, particularly in insects. In a previous field survey by Werren et al. (1995b), Wolbachia were found in more than 16% of the insects sampled. Phylogenetic analyses of the Wolbachia harbourered by various host species have shown that the Wolbachia clade can be divided into two major groups, designated A and B (Werren et al., 1995a).

Wolbachia are known to induce various reproductive alterations in their hosts, including cytoplasmic incompatibility, thelytokous parthenogenesis, feminization and male-killing (for reviews, see Werren, 1997; Stouthamer et al., 1999). Cytoplasmic incompatibility is a crossing incompatibility that results in embryonic mortality in diploid species. In haplodiploid species such as wasps, in which haploid embryos develop into males, cytoplasmic incompatibility causes male-biased sex ratios. The effect of cytoplasmic incompatibility on crossability is typically unidirectional: the cross between infected males and uninfected females is incompatible, whereas the reciprocal cross is compatible. In addition, bidirectional incompatibilities are observed when males and females carry different Wolbachia strains. It has been reported that cytoplasmic incompatibility arises from defects in paternal chromatin condensation during mitosis in Drosophila simulans (O’Neill and Karr, 1990; Lassy and Karr, 1996; Callaini et al., 1997), Nasonia vitripennis (Reed and Werren, 1995) and Culex pipiens (Jost, 1971). Cytological examinations in several species have revealed that the bacteria in spermatids are released during spermatogenesis and are absent from the mature spermatozoa (Wright and Barr, 1980; Binnington and Hoffmann, 1989; O’Neill, 1989). It is assumed that Wolbachia modify the spermatozoa during spermatogenesis and that this modification is responsible for the expression of cytoplasmic incompatibility. However, few investigations have attempted to detect the modification in the spermatozoa of Wolbachia-infected insects, mainly because it is difficult to obtain sperm samples from insects in which cytoplasmic incompatibility has been expressed.
Males of orthopteran insects release spermatophores containing pure spermatozoa when they mate. Because of this habit, samples of pure spermatozoa can be collected from the males of this insect group. However, despite this advantage, no laboratory strain of an orthopteran insect has been established as an insect model for the study of cytoplasmic incompatibility.

Masui et al. (1997) previously reported that a laboratory strain of the cricket *Teleogryllus taiwanemma* was infected with B-group *Wolbachia*, although the effect of the infection was unclear. In this study, we further investigated *Wolbachia* infection in *T. taiwanemma*. The phylogenetic position of the *Wolbachia* was estimated on the basis of the *wsp* gene that codes for a *Wolbachia* surface protein. To clarify the phenotype induced by the *Wolbachia*, we performed crossing experiments and demonstrated that cytoplasmic incompatibility is expressed, giving relatively high embryonic mortality. We also examined the presence of *Wolbachia* in various tissues of the host using the polymerase chain reaction (PCR). In addition, the bacterial density in each tissue was determined by quantitative PCR assays. After we had confirmed that the spermatozoa did not contain *Wolbachia*, we compared the profiles of sperm proteins between the infected and uninfected males.

**Materials and methods**

**Insects**

A strain of the cricket *Teleogryllus taiwanemma* infected with a B-group *Wolbachia* strain (wTai) was maintained under 16 h:8 h light:dark cycle at 25 °C. The crickets were fed an insect food purchased from Oriental Yeast, Tokyo, Japan, and provided with tap water. To establish a *Wolbachia*-free strain, the infected insects were given an aqueous solution of tetracycline hydrochloride (0.5% w/v) for two generations. This strain was reared without tetracycline for at least two generations (approximately 5 months) before being subjected to experiments.

**DNA extraction**

The antennae, heads, forewings, hindwings, testes, ovaries, Malpighian tubules, foot muscles, and fat bodies were obtained by dissection from adult insects. Haemolymph, which oozed out through the incision when a hindleg was cut, was collected in a small pipette. To obtain pure sperm samples, we cut the end of a spermatophore and immersed it in phosphate-buffered saline (PBS; 137 mmol l\(^{-1}\) NaCl, 8.1 mmol l\(^{-1}\) Na\(_2\)HPO\(_4\), 2.68 mmol l\(^{-1}\) KCl, 1.47 mmol l\(^{-1}\) KH\(_2\)PO\(_4\), pH 7.5). The spermatozoa released into PBS was centrifuged. These samples were homogenized in saline/Tris/EDTA (STE: 100 mmol l\(^{-1}\) NaCl, 10 mmol l\(^{-1}\) Tris pH 8.0, 1 mmol l\(^{-1}\) EDTA, pH 8.0) containing proteinase K at 0.4 mg ml\(^{-1}\). After incubation at 55 °C for 90 min, DNA was purified by phenol/chloroform extraction followed by ethanol precipitation.

**Phylogenetic analysis**

The *wsp* gene was amplified from the DNA solution prepared from testis by PCR using the general *wsp* primers (Braig et al., 1998) wsp\(_{81}\)F (5¢-TTGTCCAATAAGTGAA-GAAAC-3¢) and wsp\(_{69}\)IR (5¢-AAAAATTTAACGCTACT-CCA-3¢). These primers amplify a DNA fragment of approximately 600 base pairs (bp). The cycling conditions consisted of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C, and 1 min at 72 °C, and a final cycle of 5 min at 72 °C. The PCR products were directly ligated into pGEM-T vectors (Promega), and five independent clones were sequenced using an ABI PRISM 310 genetic analyzer (PE Applied Biosystems). The *wsp* sequence has been submitted to the GenBank database under accession number AB035514. This sequence was aligned to the *wsp* sequences reported by Zhou et al. (1998) and Hurst et al. (1999). A 41 bp region (positions 519–559) corresponding to the third hypervariable region of the gene (Braig et al., 1998) was omitted from the analysis because it could not be aligned with confidence (Zhou et al., 1998). Sites with gaps were also excluded from the aligned data set. The resulting alignment of approximately 500 bases was used to construct a neighbour-joining tree (Saitou and Nei, 1987) with Kimura’s two-parameter distance (Kimura, 1980) using the program package Clustal W (Thompson et al., 1994). A bootstrap test (Felsenstein, 1981) was performed with 1000 replications.

**Detection of Wolbachia**

The presence of *Wolbachia* in various tissues of *T. taiwanemma* was tested by PCR using the primer sets *ftsZ*BF (5¢-CCGATGCTCAAGCGTTAGAG-3¢) and *ftsZ*BR (5¢-CCACTTAACCTCCTTTGTT-3¢), which specifically amplify the *ftsZ* cell cycle gene of B-group *Wolbachia* (Werren et al., 1995a). The PCR cycling conditions consisted of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, and a final cycle of 5 min at 72 °C. To confirm successful DNA extraction, the samples were also subjected to PCR using primers specific for the insect *pgi* gene that codes phosphoglucose isomerase (Katz and Harrison, 1997). The primers used were *pgi*F (5¢-AACAGGAGATATGGAAATCTAATGG-3¢) and *pgi*R (5¢-TTCCAGGTTCACCCACA-3¢). The cycling conditions were the same as those for the amplification of the *ftsZ* gene.

**Quantitative PCR**

To determine the *Wolbachia* density in each tissue, quantitative PCR was performed in a LightCycler (Roche Diagnostics). A 294 bp fragment of the internal region of the *VirD4* gene (GenBank accession number AB041342) was amplified using the primers *VirD4*F (5¢-ATCAGAGAAAGACATACGAAAAGCAGG) and *VirD4*R (5¢-CAATGCTTACCCCATGCTGCC). The 20 μl reaction mixture consisted of 10% (v/v) LightCycler DNA master SYBR Green I (Roche Diagnostics), 3 mmol l\(^{-1}\) MgCl\(_2\), 0.2 mmol l\(^{-1}\) of each primer, 2 μl of template DNA and 0.16 μl of TaqStart Antibody (Clontech). For the amplification, 50 cycles consisting of 0 s at 95 °C, 5 s at 57 °C and 30 s at 72 °C were performed. The fluorometric intensity of SYBR Green I, a dye specific for double-stranded DNA, was measured at the end of each elongation phase, and the original concentration of the *VirD4*
gene in the template DNA solution was determined by analysis of the amplification kinetics. Standard reactions were performed with the template solutions containing VirD4 PCR products at known concentrations. The primer set used amplified the VirD4 gene without production of primer dimers or non-specific background, which was confirmed by a melting-curve analysis as recommended by the manufacturer. On the basis of the observation that the VirD4 gene of wTai is a single copy gene (S. Masui, unpublished data), the number of Wolbachia cells in each sample was calculated from the concentration of the gene.

**Crossing experiments**

Crossing experiments were performed using single pairs of virgin individuals. Females and males were separated at the late nymphal stage; they were paired on adult emergence and kept in a cage with a 50 ml tube containing moistened tissue paper. Approximately 1 week after they had been paired, the female laid eggs on the tissue paper. The eggs were washed off the tissue paper and placed onto moistened filter papers in a plastic dish. More than 100 eggs were collected from each pair to count the number of hatched nymphs. After the eggs had been collected, all individuals used in the experiment were diagnosed by the PCR using the ftsZB primers to confirm the infection status. The DNA samples were prepared from the testes or ovaries.

**Electrophoresis of sperm proteins**

Spermatzoa were dissolved in 240 μl of the lysis buffer [9.8 mol l⁻¹ urea, 0.5 % (v/v) Triton-X, 2 % Amphilone, (pH 3.5–10), 0.075 % (w/v) dithiothreitol and a small amount of Orange G], then applied to gels (Immobiline DryStrip, pH 3–10, 11 cm; Amersham Pharmacia Biotech). The gels were rehydrated for 12 h and then subjected to electrophoresis in a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech). Isoelectric focusing was performed at 15 °C with pH 3–10, 11 cm; Amersham Pharmacia Biotech). The gels were rehydrated for 12 h and then subjected to electrophoresis in a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech). Isoelectric focusing was performed at 15 °C with voltage limits of 300 V for 1 h, a gradient from 300 V to 3500 V for 3 h, and 3500 V for 17 h. The current limit was 0.5 mA per strip throughout the electrophoresis. In the second dimension, SDS-PAGE was performed as described by Laemmli (1970) using slab separation gels of 10 % polyacrylamide. The proteins were visualized by silver staining using a two-dimensional silver stain II ‘DAIICHI’ (Daichi Pure Chemicals). The protein profiles were compared between infected and uninfected males by superimposing the two gels on a light box.

**Results**

**Phylogenetic relationship**

To estimate the relative phylogenetic position of Wolbachia infecting T. taiwanemama (wTai), the wsp gene was sequenced and aligned to those previously reported from other host species. The phylogenetic analysis suggested that wTai is closely related to Wolbachia strains infecting the small brown planthopper Laoedphax striatellus (wStri), the flower beetle Tribolium confusum (wCon), the butterfly Acraea encedon (wEnc), the wasp Trichogramma deion (wDei) and the ladybird Adalia bipunctata (wBip, wBip).

<table>
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<tr>
<th>Cross</th>
<th>Number of crosses</th>
<th>Number of eggs collected</th>
<th>Number of eggs hatched</th>
<th>Percentage of eggs hatched</th>
</tr>
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<tbody>
<tr>
<td>Infected × infected</td>
<td>9</td>
<td>1497</td>
<td>1282</td>
<td>82.0±10.9</td>
</tr>
<tr>
<td>Infected × uninfected</td>
<td>15</td>
<td>2712</td>
<td>861</td>
<td>20.3±18.7</td>
</tr>
<tr>
<td>Uninfected × infected</td>
<td>5</td>
<td>667</td>
<td>553</td>
<td>82.1±10.4</td>
</tr>
<tr>
<td>Uninfected × uninfected</td>
<td>8</td>
<td>1067</td>
<td>920</td>
<td>86.7±7.1</td>
</tr>
</tbody>
</table>

*Mann-Whitney U-test (compared with all other crosses): P<0.001.*

Values are means ± S.D.

Wolbachia infection in cricket

**Crossing experiments**

The expression of cytoplasmic incompatibility, a possible reproductive alteration induced by the Wolbachia, was examined by crossing experiments between the infected insects and those from which the Wolbachia had been removed by tetracycline treatment. When uninfected females were mated with infected males, the egg hatch rates were 0–48.6 %, with a mean rate of 20.3 % (Table 1). In each of the other three crosses, more than 80 % of the eggs hatched. The egg hatch rate in the crosses between infected males and uninfected females was statistically different from those in the other combinations (Mann–Whitney U-test, P<0.001). The duration of embryonic development was approximately 3 weeks at 25 °C. In the compatible crosses, the eggs swelled within a few days of oviposition. Most eggs from the incompatible cross did not swell, suggesting that embryonic death had occurred at a relatively early stage of the development.

**Distribution of Wolbachia**

To examine the distribution of Wolbachia in T. taiwanemama, DNA was prepared from the antennae, heads, forewings, hindwings, testes, ovaries, Malpighian tubules, foot muscles, fat bodies, haemolymph and spermatooza. In the PCR assay using ftsZB primers, we detected Wolbachia in all samples examined, except in the haemolymph and spermatooza (Fig. 2A). The success of the DNA extraction was confirmed by PCR using pgi primers (Fig. 2B).

We also performed quantitative PCR to determine the densities of Wolbachia in the tissues in which the bacteria had been detected. The total number of bacteria per tissue was highest in the ovaries (480×10⁶ bacteria) and relatively high in the fat bodies, testes and heads. The bacterial density, estimated by normalising the total bacterial number by the wet mass for each tissue, was highest in the fat bodies.
The absence of Wolbachia in the spermatozoa of the infected T. taiwanemma suggested that the Wolbachia modified the spermatozoa to induce cytoplasmic incompatibility during sperm development. For this reason, any difference between the spermatozoa of infected and uninfected males should have been related to the expression of cytoplasmic incompatibility.

Analysis of sperm proteins

For this reason, any difference between the spermatozoa of infected and uninfected males should have been related to the expression of cytoplasmic incompatibility.

Discussion

Reproductive incompatibilities have been reported in allopatric field populations of Gryllus crickets (Harrison, 1983;
Smith and Cade, 1987; Cade and Tyshenko, 1990). Recently, some of them were found to be infected with Wolbachia (Werren et al., 1995a; Giordano et al., 1997). In the present study with *T. taiwanemma*, we generated a Wolbachia-free strain from the infected strain to perform crossing experiments, and observed that the cross between the infected males and uninfected females was incompatible, giving an embryonic mortality at 79.7%. To our knowledge, this is the first clear-cut evidence for the association between mortality and uninfected females was incompatible, giving an embryonic and observed that the cross between the infected males and uninfected females was incompatible, giving an embryonic mortality at 79.7%. To our knowledge, this is the first clear-cut evidence for the association between Wolbachia infection and unidirectional cytoplasmic incompatibility in an orthopteran insect. It is known that the strength of cytoplasmic incompatibility varies depending on the host species. For example, Wolbachia induce almost complete incompatibility in the almond moth *Ephestia cautella* (Sasaki and Ishikawa, 1999), in *Laodelphax striellus* (Noda, 1984) and in the genus *Tribolium* (Stevens and Wade, 1990), while *Drosophila melanogaster* shows weak expression of cytoplasmic incompatibility with an embryonic mortality lower than 30% (Hoffmann, 1988; Hoffmann et al., 1994). In comparison with other host species, the strength of cytoplasmic incompatibility observed in *T. taiwanemma* may be considered as intermediate or relatively high.

Phylogenetic analysis based on the wsp gene (Fig. 1) suggested that wTai is phylogenetically close to wStri, wCon, wEnc, wDei, wBipA and wBipB. Interestingly, these Wolbachia strains induce different reproductive phenotypes: wStri and wCon induce cytoplasmic incompatibility, wDei causes thelytokous parthenogenesis (Stouthamer et al., 1993) and the others are male-killers (Hurst et al., 1999). Thus, closely related Wolbachia have different effects on their host, as was also described in a recent study by Van Meer et al. (1999). One explanation for the lack of congruence between Wolbachia phylogeny and their phenotypes is that the host, rather than Wolbachia, plays a major role in the determination of the phenotype. It is also possible that Wolbachia easily acquire the ability to induce various effects. Different phenotypes may evolve rapidly if the genes responsible for them are similar or if these genes are located on a transposable element that can move between strains. It has been demonstrated that the genome of wTai contains transposon-like sequences (Masui et al., 1999) and phages (S. Masui, unpublished data).

In the PCR assay using ftsZB primers, Wolbachia were detected in all the samples examined, except in the spermatozoa and haemolymph (Fig. 2). Since Wolbachia are maternally transmitted through the egg cytoplasm and induce reproductive alterations, it has been assumed that they infect the reproductive tissues of the hosts. Contrary to this assumption, we have shown here that Wolbachia are widely distributed in the tissues of *T. taiwanemma*, a finding consistent with the previous reports on Wolbachia infections in *Drosophila simulans*, the mosquitoes *Aedes albopictus* and *Culex pipiens*, the moth *Ephestia cautella* (Dobson et al., 1999) and the isopod *Armadillidium vulgare* (Martin et al., 1973). In the present study, we further examined the somatic infection of Wolbachia in terms of bacterial density. The quantitative PCR assays (Table 2) showed that the density in fat bodies was higher even than that in the ovaries or testes. The bacterial density differed greatly among tissues, suggesting that some tissues, such as the muscle and brain, are not suitable for the proliferation of wTai. The distribution and density of Wolbachia in the tissues of adult insects may also be related to the bacterial localization in the host egg, which has not yet been examined for *T. taiwanemma*.

To explain the mechanism of cytoplasmic incompatibility, it has often been postulated that Wolbachia secrete a protein that remains in mature spermatozoa (Hurst, 1991; Lassy and Karr, 1996). When Sasaki et al. (1998) analyzed sperm proteins from *Drosophila simulans*, no protein specific to the infected flies was detected. Compared with *Drosophila* spp., *T. taiwanemma* has some features suitable for sperm analysis: (i) mature spermatozoa can be obtained easily from spermatophores without contaminating the spermatids; (ii) spermatophores can be collected from a male repeatedly without killing it; and (iii) spermatophores contain sufficient...
volumes of spermatozoa for biochemical and molecular biological analyses. In this study, we took advantage of these features of *T. taiwanenmensa* to analyze larger numbers of spermatozoa and performed isoelectric focusing over a wider range than in the previous study with *D. simulans*. In spite of these improvements in the methodology, we were still unable to detect any repeatable difference in the protein profiles between the infected and uninfected individuals. This could mean that the amount of the protein involved in cytoplasmic incompatibility expression is extremely small or that the pl and/or molecular size of the protein are out of range of the two-dimensional gel system employed. It is also possible that cytoplasmic incompatibility is caused by such a small modification in protein structure that it could not be detected. Alternatively, the molecule responsible for cytoplasmic incompatibility may not be a protein, but some other molecule, such as a nucleic acid or a compound of low molecular mass. To examine these possibilities, further experiments are needed.

**References**


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