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Evidence for a *Wolbachia* symbiont in *Drosophila melanogaster*

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Summary

The bacterial cell division gene, *ftsZ*, was used as a specific probe to show the presence of a symbiotic bacterium in two wild type strains of *Drosophila melanogaster*. Under stringent hybridization conditions we have shown that the bacterium is transferred to the progeny of these strains from infected mothers and can be eradicated by treatment with the antibiotic tetracycline. We have characterized this bacterium, by amplifying and sequencing its 16S rRNA gene, as being a member of the genus *Wolbachia*, an organism that is known to parasitize a range of insects including *Drosophila simulans*. In a series of reciprocal crosses no evidence was found that the symbiont causes cytoplasmic incompatibility (CI) which is known to occur in infected strains of *D. simulans*. The implications of these findings are discussed.

1. Introduction

Cytoplasmic incompatibility (CI) exists in a wide range of insects and is caused by the presence of an intracellular bacterial organism that is transmitted maternally to the offspring (Hoffmann *et al.* 1986; Stevens & Wade, 1990). In most cases, CI results from a cross between an infected male and an uninfected female which produces eggs that fail to hatch. Bidirectional CI, where both adults are infected, has also been reported in insect populations although to a lesser degree (O'Neill & Karr, 1990). The organisms that cause CI have been characterized as members of the genus *Wolbachia*, a *Rickettsial*-like bacterium that was first observed and described in the mosquito *Culex pipiens* (Hertig, 1936).

Wolbachia is known to exist in some *Drosophila* species where it is known to cause both bi- and unidirectional CI (Turelli & Hoffmann, 1991; O'Neill *et al.* 1992). Although it cannot be cultured outside the host organism the organism can be detected with the use of electron microscopy and fluorescent stains (Louis & Nigro, 1989; O'Neill & Karr, 1990). In this way it has been observed to infect and disintegrate the nuclei of the developing zygote, eventually causing the embryos to die (O'Neill & Karr, 1990). Eradication of these parasites from insect tissue has been made possible with the use of the antibiotic tetracycline; this produces cured insects that can be used in reciprocal crosses to determine the cause and nature of parasite transmission (Hoffmann *et al.* 1986; O'Neill & Karr, 1990). Lately, with the use of molecular techniques the characterization of *Wolbachia* in *Drosophila simulans* as well as in many other insects has become more circumstantial (O'Neill *et al.* 1992; Rousset *et al.* 1992). PCR amplification technology coupled with bacterial 16S rRNA sequence data has provided a quick and efficient way of determining the presence and type of parasite. Furthermore, this analysis has enabled the phylogenetic classification of the parasite within the Eubacteria as well as showing that *Wolbachia* is more pervasive among insect populations than originally thought (O'Neill *et al.* 1992).

There is evidence to show that *Wolbachia* is likely to exist in strains of *D. melanogaster* (Wolstenholme, 1965; Hoffmann, 1988; Glover *et al.* 1990). Hoffmann demonstrated partial CI between two Australian strains of *D. melanogaster* and showed that the causative factor was maternally inherited over three generations and could be eliminated with tetracycline. He proposed that the possible causative agent of the incompatibility was *Wolbachia*. Another report describes tetracycline-sensitive parasites in young embryos of a *D. melanogaster* strain by showing clusters of DAPI-stainable spots under the microscope; however the type of organism and its relative effects

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on CI were not determined (Glover *et al.* 1990). Recently, we have been successful in characterizing the cell division gene *ftsZ* from *Wolbachia* in a wild type strain of *D. melanogaster*, providing the first molecular evidence of *Wolbachia* in *D. melanogaster* (Holden *et al.* 1993).

In this paper we use the *ftsZ* gene as a *Wolbachia*-specific probe to determine the presence and sensitivity of the bacterium and its method of transfer from parents to offspring in wild type strains of *D. melanogaster*. Furthermore, by using infected and uninfected strains we examine the effect of the contaminant on CI and compare these results with those obtained from an earlier study made by Hoffmann (1988).

2. Methods and Materials

(i) *Origin and treatment of fly strains*

The three *D. melanogaster* wild type strains utilized were Canton-S, Harwich and Monty-12. Of these Canton-S and Harwich are long established inbred laboratory strains, supplied to us by Dr B. Burnet (University of Sheffield). Monty-12 is one of a collection of isofemale lines collected in 1986 near Montpellier, France, and subsequently inbred by 30 generations of sib mating. Crosses and reciprocal crosses were performed as follows:

1. Canton-S^m × Canton-S^f. 2. Canton-S^m × Monty-12^f.

3. Monty-12^m × Canton-S^f. 4. Monty-12^m × Monty-12^f.

The crosses were performed by taking individual pairs of one male and one female and leaving them in 7·5 g of standard cornmeal/treacle/yeast medium (75 g maizemeal, 11 g flaked yeast, 10 g agar, 75 g treacle, 2·5 ml propionic acid, 2·5 g nipagin and make up to 1 l with water). We attempted to randomize the parental flies for size and age across the crosses. Vials were kept at 22 °C and the parents were removed after 10 days. After 25 days the F1 flies were counted and stored at −80 °C for DNA extraction.

For tetracycline treatment adult flies were reared on the standard medium containing 0·25 mg/ml tetracycline for three weeks. The second generation of flies were transferred to fresh medium containing 1 mg/ml tetracycline for five days before being transferred back to their normal growth medium without tetracycline. Flies taken for DNA extraction were stored at −80 °C for at least 6 h prior to any manipulation.

(ii) *DNA extraction and Southern Blot analysis*

Total genomic DNA was extracted from adults and offspring of the three *Drosophila melanogaster* wild type strains using the procedure described by Ish–Horowicz (Protocol 47) in Ashburner (1989). Around 5 μg of RNase-treated genomic DNA was *Eco*RI digested and electrophoresed through 0·8% agarose (Ultra Pure, BRL, USA) using 1 × TBE as the buffer. The DNA was depurinated with 0·25 M-HCl for 7 mins, denatured with 0·5 M-NaOH/1·5 M-NaCl for 30 mins then renatured with 0·5 M-Tris/HCl/1·5 M-NaCl for 30 mins. The DNA was transferred to a nylon filter by capillary blotting then dried and cross-linked. Hybridization was carried out at 65 °C using a digoxygenin-labelled probe which was prepared according to the manufacturer's recommendations (Boehringer-Mannheim, Germany). A cloned 2·7 kb DNA fragment containing the 1·2 kb *ftsZ* gene and a cloned 1180 bp PCR-derived 16S rRNA gene were both labelled and used as probes. This was followed by 2 × 5 min washes with 2 × SSC/0·5% SDS and 2 × 15 min washes with 0·1 × SSC/0·5% SDS prior to detection. Hybridization was detected using an antibody-enzyme conjugate and visualized with an alkaline phosphatase colour reaction.

(iii) *Amplification of 16S rRNA gene*

Selective PCR amplification was carried out using Eubacterial-specific 16S rRNA primers on genomic DNA from infected and tetracycline-treated flies. The primers used in the PCR were as follows: 5′ GGACCGGATCCGCTTAACACATGCAAG 3′ (*E. coli* position 45-61 forward) and 3′ GGACCGAATTCCCATTGTAGCACGTGT 5′ (*E. coli* position 1242-1227 reverse) (O'Neill *et al.* 1992). An *Eco*RI restriction site was added to each primer for cloning purposes. The conditions for the PCR were as follows: denaturation for 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 40 s, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The final reaction product was extracted with phenol/chloroform and precipitated with iso-propanol. The DNA was then *Eco*RI-digested for 1 h at 37 °C then precipitated with iso-propanol prior to cloning.

(iv) *Cloning and sequencing the 16S rRNA gene*

The 1180 bp PCR-derived 16S rRNA gene was cloned directly into the *Eco*RI site of the cloning vector pGEM7Zf(+) (Promega, USA). The cloning was carried out according to the manufacturer's recommendations using DH5α as the recipient host strain. This clone was used to prepare double stranded sequencing template (Sambrook *et al.* 1989). DNA sequencing using the dideoxynucleotide chain termination method was carried out with the Sequenase 2·0 kit (U.S. Biochemicals) using the T7 or SP6 primer from the vector.

(v) *DNA sequence and statistical analysis*

GenBank database homology searches using the FASTA program and DNA sequence comparisons

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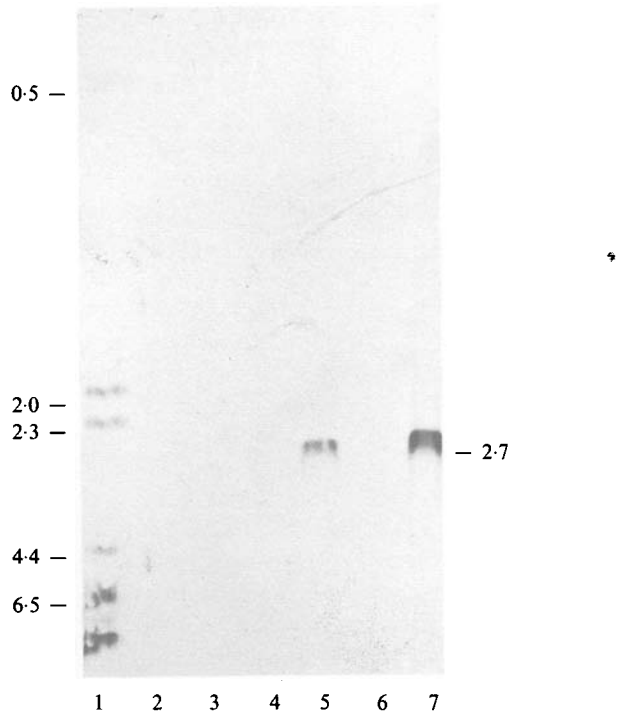

Fig. 1. Southern blot analysis of *Eco*RI-digested genomic DNA extracted from tetracycline-treated (TT) and untreated flies using the digoxygenin-labelled 2·7 kb clone containing the *fts*Z gene as a probe. The DNA was run as follows; 1. λ Hind III marker, 2. (TT)-Monty-12, 3. Monty-12, 4. (TT)-Harwich, 5. Harwich, 6. (TT)-Canton-S, 7. Canton-S. Sizes of molecular weight markers are indicated in Kb.

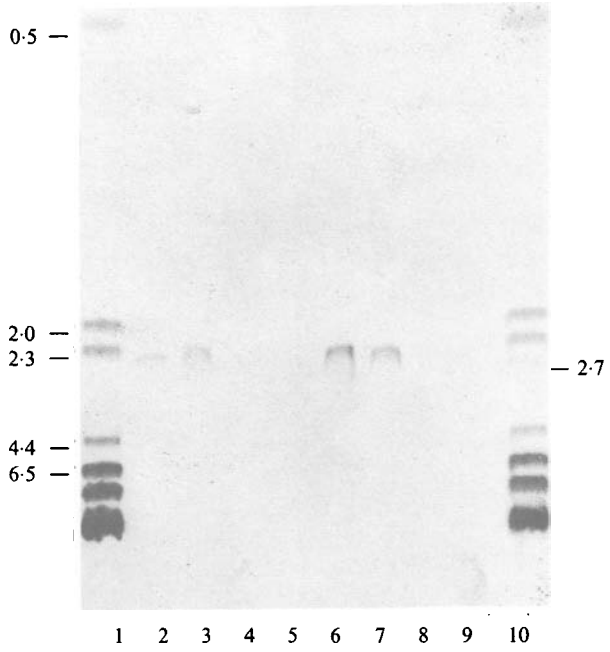

Fig. 2. Southern blot analysis of *Eco*RI-digested genomic DNA extracted from male (m) and female (f) flies of parents and F1 progeny of a reciprocal crossing experiment between infected Canton-S (C-S) and uninfected Monty-12 (M-12) strains using the digoxygenin-labelled *fts*Z gene as a probe. The DNA was run as follows; 1 and 10 λ Hind III marker, 2. Canton-S^m, 3. Canton-S^f, 4. Monty-12^m, 5. Monty-12^f, 6. Cross $(M\text{-}12^m \times C\text{-}S^f)^m$, 7. Cross $(M\text{-}12^m \times C\text{-}S^f)^f$, 8. Cross $(M\text{-}12^f \times C\text{-}S^m)^m$, 9. Cross $(M\text{-}12^f \times C\text{-}S^m)^f$. Sizes of molecular weight markers are indicated in Kb.

were carried out using the University of Wisconsin Genetics Computer Group programs (Devereux *et al.* 1984; Lipmann & Pearson, 1985). Significant differences in the number of progeny derived from the reciprocal crossing experiment were determined statistically. The number of offspring per cross was treated as a normally distributed parametric variable and pairwise comparisons between the four crosses were performed using *t*-tests.

3. Results

(i) *Evidence of a bacterium in* D. melanogaster

Southern blot analysis was carried out using *Eco*RI-digested genomic DNA from three wild type strains of *D. melanogaster* (Canton-S, Harwich and Monty-12) that had been treated with or without the antibiotic tetracycline. Using the bacterial gene *fts*Z as a specific probe we were able to detect the bacterium in our untreated Canton-S and Harwich strains (Fig. 1). The probe hybridized to a 2·7 kb fragment in DNA from these flies indicating that the bacterial DNA had been removed by the antibiotic in the treated flies (Fig. 1). The probe failed to hybridize to DNA preparations from treated and untreated Monty-12 indicating that this strain was not a host to this *fts*Z-specific bacterium (Fig. 1).

(ii) *The bacterium is maternally inherited*

We decided to test whether the bacterium was maternally transferred to the offspring in our infected strains. To show this we had to assume that infected offspring would only result from a cross with infected females. Reciprocal crosses were set up using the infected (Canton-S) and uninfected strains (Monty-12). *Eco*RI-digested genomic DNA from both male and female parents as well as the progeny from the reciprocal crosses was blotted and probed with *fts*Z (Fig. 2). The probe hybridized to DNA, indicating the presence of a bacterium, from both the male and female Canton-S parents and the progeny of the cross between Canton-S females and Monty-12 males ($C\text{-}S^f \times M\text{-}12^m$) (Fig. 2). Bacterial DNA was not found in Monty-12 parent flies or in the progeny of the other reciprocal cross (Fig. 2). This shows conclusively that the bacterium is transmitted maternally from adults to offspring in our infected wild type strains of *D. melanogaster*.

(iii) *Characterization of the bacterium using 16S rRNA analysis*

Amplification of specific genes by the polymerase chain reaction (PCR) has been the usual route taken

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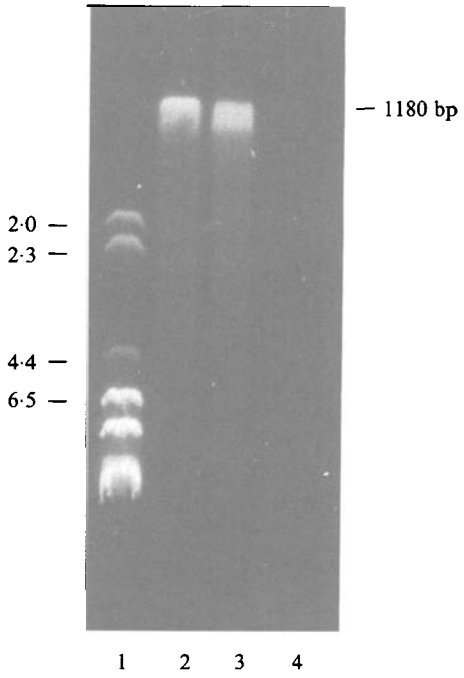

Fig. 3. An ethidium stained gel showing the 1180 bp PCR product amplified using 16S rRNA specific primers on genomic DNA derived from different fly strains. The DNA was run as follows; 1. λ Hind III marker 2. Canton-S 3. Harwich 4. Tetracycline-treated Canton-S. Sizes of molecular weight markers are indicated in Kb.

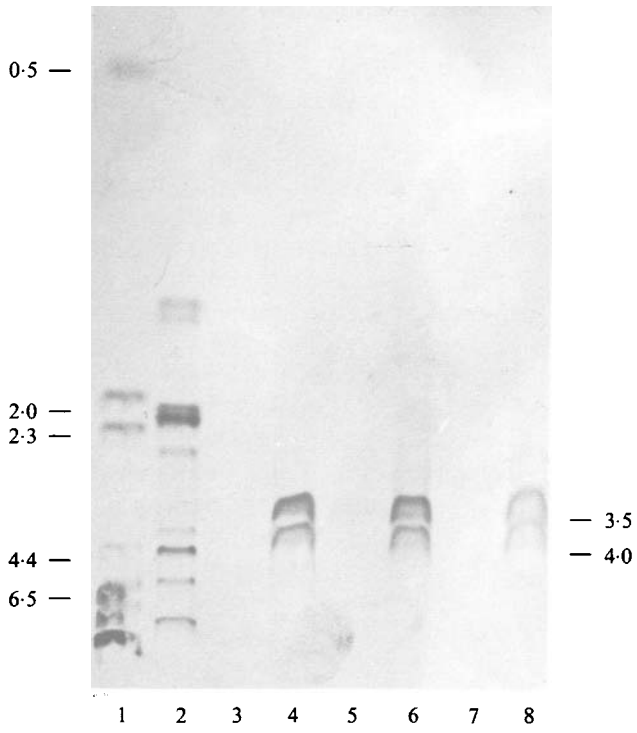

Fig. 4. Southern blot analysis of *Eco*RI-digested genomic DNA extracted from tetracycline treated (TT) and untreated flies using the digoxygenin labelled 1180 bp 16S rRNA gene as a probe. The DNA was run as follows; 1. λ Hind III marker, 2. *E. coli* control, 3. (TT)-Canton-2, 4. Canton-S, 5. (TT)-Harwich, 6. Harwich, 7. (TT)-Cross $(M\text{-}12^{m} \times C\text{-}S^{f})^{m}$, 8. Cross $(M\text{-}12^{m} \times C\text{-}S^{f})^{m}$. Sizes of molecular weight markers are indicated in Kb.

to circumvent the problems that arise in identifying bacterial parasites of insects. We decided to identify the bacterium by amplifying then sequencing the bacterial-specific 16S rRNA gene directly from genomic DNA. Using Eubacterial-specific primers we were successful in amplifying the predicted 1180 bp product from genomic DNA of our untreated Canton-S and Harwich strains (Fig. 3). Our attempts to amplify the gene from treated Canton-S DNA was unsuccessful apart from a faint amplification product which would result from small amounts of residual bacterial DNA remaining after antibiotic treatment (Fig. 3). Using the Canton-S 1180 bp PCR product as a probe we were able to show the presence of the bacterium in DNA from untreated flies (Fig. 4). In this respect the probe hybridized to two *Eco*RI-restricted fragments of genomic DNA from untreated Canton-S and Harwich strains as well as infected offspring from one of the reciprocal crosses (Fig. 4). This restriction pattern illustrates the presence of an *Eco*RI site which has already been mapped within the gene (Fig. 4). This probe failed to hybridize to DNA extracted from any of the cured flies, suggesting that the gene and bacterium had been removed with tetracycline treatment (Fig. 4).

We sequenced part of the cloned 1180 bp PCR product derived from Canton-S and submitted a 204 bp sequence to the GenEMBL database. The search revealed that the DNA was identical to part of the 16S rRNA gene of the *Rickettsia*-related Proteobacterium *Wolbachia* found in the Hawaii strain of *Drosophila simulans* (Rousset *et al.* 1992) (Fig. 5). Alignments also revealed that the 204 bp sequence was almost identical, a one base difference, to part of the 16S rRNA gene of *Wolbachia pipiensis* in the Riverside strain of *D. Simulans* (O'Neill *et al.* 1992) (Fig. 5). This provides conclusive evidence that the 1180 bp PCR product is part of the 16S rRNA gene of a *Wolbachia* contaminant of *D. melanogaster*.

(iv) *Cytoplasmic incompatibility is not found in infected strains*

We decided to test for unidirectional CI in our fly strains. To do this we had to assume that unidirectional CI would only occur when infected males were crossed with uninfected females. The progeny from ten replicate crosses, set up between individual male and female flies within and between our *Wolbachia*-infected (Canton-S) and uninfected (Monty-12) strains, were counted to test for CI (Table 1). The results showed that there were no significant differences in the number of progeny produced from the ten replicate crosses of the Monty-12 female and Canton-S male cross (Table 1). In fact there were no significant differences between all four crosses, where it was found that all matings produced a large number of viable progeny (Table 1). This result provides evidence to suggest that the contaminating *Wolbachia*

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D.melanogaster (CantonS)   1   ggtgagtaatgtataggaatctacctagtagtacggaataattgtt 46
D.simulans (Hawaii)        39  ggtgagtaatgtataggaatctacctagtagtacggaataattgtt 84
D.simulans (Riverside)     51  ggtgagtaatgtataggaatctacctagtagtacggaataattgtt 96

D.melanogaster (CantonS)   47  ggaaacggcaactaataccgtatacgccctac.ggggaaaaattta 91
D.simulans (Hawaii)        85  ggaaacggcaactaataccgtatacgccctac.ggggaaaaattta 129
D.simulans (Riverside)     97  ggaaacggcaactaataccgtatacgccctacgggggaaaaattta 142

D.melanogaster (CantonS)   92  ttgctattagatgagcctatattagattagctagttggtggagtaa 137
D.simulans (Hawaii)        130 ttgctattagatgagcctatattagattagctagttggtggagtaa 175
D.simulans (Riverside)     143 ttgctattagatgagcctatattagattagctagttggtggagtaa 188

D.melanogaster (CantonS)   138 tagcctaccaaggcaatgatctatagctgatctgagaggatgatca 183
D.simulans (Hawaii)        174 tagcctaccaaggcaatgatctatagctgatctgagaggatgatca 221
D.simulans (Riverside)     189 tagcctaccaaggcaatgatctatagctgatctgagaggatgatca 234

D.melanogaster (CantonS)   184 gccacactggaactgagatac 204
D.simulans (Hawaii)        223 gccacactggaactgagatac 242
D.simulans (Riverside)     236 gccacactggaactgagatac 255
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Fig. 5. A 204 base pair DNA alignment of the 16S rRNA gene from *Wolbachia* symbionts of one wild type strain of *D. melanogaster* (Canton-S) and two strains of *D. simulans* (Hawaii and Riverside).

Table 1. *Number of progeny resulting from ten replicate crosses of four matings (m = male, f = female) between an infected Canton-S strain (Can) and an uninfected Monty-12 strain (Mon). The mean and standard deviation (S.D.) are given along with the results of the statistical t-test*

Mating Rep	a $Can^f \times Can^m$	b $Can^f \times Mon^m$	c $Mon^f \times Can^m$	d $Mon^f \times Mon^m$
1	24	4	10	17
2	15	26	30	28
3	12	33	23	23
4	25	30	20	23
5	3	36	28	—
6	34	9	27	25
7	24	26	18	24
8	38	27	31	15
9	88	17	6	11
10	37	8	29	21
Mean	30·0	21·6	22·2	20·8
S.D.	23·3	11·3	8·7	5·4

Results of pairwise comparisons

$a \times b$ $t = 1{\cdot}05$ n.s. $a \times c$ $t = 0{\cdot}99$ n.s.
$a \times d$ $t = 0{\cdot}15$ n.s. $b \times c$ $t = 0{\cdot}15$ n.s.
$b \times d$ $t = 0{\cdot}19$ n.s. $c \times d$ $t = 0{\cdot}41$ n.s.

The differences between means were not-significant.

strain was causing little or no CI in our *D. melanogaster* strains.

4. Discussion

Advances made in 16S rRNA sequence analysis to determine the presence and type of *Wolbachia* parasites in insects indicate that the bacteria are widespread and all members of the same species, *Wolbachia pipiensis*, a *Rickettsial*-like organism that belongs to the α-subdivision of the Proteobacteria (Ochman & Wilson, 1987; O'Neill *et al.* 1992; Rousset *et al.* 1992). We have shown that a bacterial symbiont is present in two wild type strains of *D. melanogaster*. Furthermore, by cloning and sequencing part of the 16S rRNA gene from the contaminant from one of these strains we

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have been able to determine that it is a member of the genus *Wolbachia*.

The product of the bacterial gene *ftsZ* that codes for a cell division protein has been characterized in *Wolbachia* and shown to be well conserved among the Eubacteria (Holden *et al.* 1993). The gene has provided an ideal bacterial probe to detect the presence of the bacterium in our *D. melanogaster* strains. In this way we have shown that it is present in both Canton-S and Harwich wild type strains but absent from another strain, Monty-12. Furthermore, we have shown that it can be eradicated from infected flies by allowed them to grow on medium supplemented with tetracycline. These results would support the findings of tetracycline-sensitive commensal parasites within other strains of *D. melanogaster* (Hoffmann, 1988; Glover *et al.* 1990). Based on his findings, Hoffmann (1988) postulated that the contaminant of his Melbourne and Townsville strains was *Wolbachia* and recent evidence identifying the organism in *D. simulans* supports these findings (O'Neill *et al.* 1992; Rousset *et al.* 1992). The results from this paper provide conclusive molecular evidence that *Wolbachia* exists in two *D. melanogaster* wild type strains, giving more support to Hoffmann's original findings (1988).

Cytoplasmic incompatibility (CI) has been shown to be caused by an intracellular and maternally-transmitted bacterium (Hoffmann, 1988; Stevens & Wade, 1990). In support of the latter characteristic we have shown that the *Wolbachia* contaminant is only transferred to the offspring from the infected mother. Although we do not present visual evidence we would assume from these results that the bacterium is intracellular, similar to the cytoplasmically-located commensal parasites observed in young embryos of *D. melanogaster* (Glover *et al.* 1990). These informative results obtained by DAPI-staining have revealed the presence of non-disruptive intracellular parasites around the centrosomes and mitotic spindles in 0–2 h old embryos (Glover *et al.* 1990).

CI causes embryonic death or sex ratio distortions and is known to occur in a wide range of insect species. Uni- and bidirectional CI leads to cell division defects in developing embryos of *D. simulans* (O'Neill & Karr, 1990. Unidirectional CI is more prevalent and results from a cross between an infected male and uninfected female. The direct cause of this phenomenon is unknown although it is likely to be due to defects in the structure and function of the sperm during fertilization (O'Neill & Karr, 1990). In contrast to the observations found in *D. simulans* we have shown that CI is not present in our infected *D. melanogaster* strains. In this respect we did not observe a failure of eggs to hatch or a low offspring number when we crossed our infected Canton-S males with uninfected Monty-12 female flies. In fact the progeny numbers were not significantly different from a normal cross between two infected or uninfected strains. Hoffmann (1988) has already looked at incompatibility in *D. melanogaster* strains and reported that partial CI (reduced hatchability) was present. The results presented here would support the assumption that *D. melanogaster* is fully or partially resistant to the incompatibility effects of contaminating *Wolbachia*. The reasons for this are not known; it is possible that *D. melanogaster* is exerting an unknown pressure on the symbiont and in turn disabling it from its normal mode of action. Any possibility that the absence of CI is due to a different contaminant has to be discounted on the basis of the results obtained by the 16S rRNA gene sequencing. Indeed, the occurrence of non-CI *Wolbachia* contaminants has been reported in other insects and also suggests that the symbiont is likely to be more prevalent than originally thought (Hurst *et al.* 1992; O'Neill *et al.* 1992).

There is increasing evidence to support the presence of a single species of bacterium within contaminated insects. Sequence analysis of the 16S rRNA gene from the bacterial symbiont of six insect species, as well as two different lines of *D. simulans*, has shown that they are all very well conserved (O'Neill *et al.* 1992). This all provides evidence that the symbionts including the bacterium from *D. melanogaster* should be classified as members of the same species, *Wolbachia pipiensis*. It would also appear on the basis of our results and others that this species does not always cause CI in insects, discounting the original idea that CI was a general reaction to infection by a range of bacterial contaminants. Furthermore, the ubiquity of this species supports horizontal rather than vertical transmission of bacterial symbionts within and between insect populations (Hurst *et al.* 1992).

In contrast, to these results, we also found that one of the *D. melanogaster* wild type strains, Monty-12, did not harbour the bacterium, The reason for its absence is unknown but might suggest that the two contaminated strains were infected before their arrival in this laboratory, unless Monty-12 has developed an inherent resistance to the parasite.

These results have highlighted the possibility of using both CI and non-CI infected strains of *Drosophila* as models for understanding the mechanisms of spread within insect populations as a whole. Furthermore, the pervasive nature of CI-causing parasites and the speed at which infection spread within a population has caused some debate on its potential role in manipulating insect populations (Turelli and Hoffmann, 1991, Curtis, 1992).

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