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The diversity of inherited parasites of insects: the male-killing agent of the ladybird beetle *Coleomegilla maculata* is a member of the Flavobacteria

GREGORY D. D. HURST[1]*, TANSY C. HAMMARTON[1], CLAUDIO BANDI[2], TAMSIN M. O. MAJERUS[1], DOMINIQUE BERTRAND[1] AND MICHAEL E. N. MAJERUS[1]
[1] *Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK*
[2] *Istituto di Patologia Generale Veterinaria, Università di Milano, Via Celoria 10, 20133 Milan, Italy*

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Summary

Many species of insects bear maternally inherited bacteria which contribute to the physiology and metabolism of their host whereas others bear inherited bacteria which are regarded as parasitic. Parasitic behaviours centre on disruption of the production, survival or fertility of male hosts, through which the microbe is not transmitted. One of the most common of these is early male-killing, where the bacteria kill male embryos they enter. The question arises as to why all inherited symbionts are not male-killers. The male-killing agents identified to date derive from two groups: the Proteobacteria and the Mollicutes. However, bacteria from other groups (Spirochaetes, Flavobacteria) are found to be inherited in insects, but contain no incidence of male-killing. We show here an association of male-killing with a bacterium from one of these groups. The bacterium causing male-killing behaviour in the ladybird *Coleomegilla maculata* is most closely related to *Blattabacterium*, a host-beneficial Flavobacteria found in cockroaches and some termites. This result indicates there is little if any taxonomic bar to the evolution of male-killing behaviour. This pattern severely contrasts with the evolution of two other parasitic symbiont behaviours, cytoplasmic incompatibility and the induction of host parthenogenesis, which have been found to be associated exclusively with bacteria from one group: *Wolbachia*. The result is briefly discussed in the light of the incidence of parasitic and beneficial cytoplasmic elements among insects.

1. Introduction

More than 10% of insect species are thought to bear maternally inherited symbionts. These are bacteria, fungi and viruses which either live inside cells and are transovariolly transmitted, or live outside cells and are transmitted transovum (Buchner, 1965). These symbionts are in an interesting evolutionary position. Being maternally inherited, selection favours symbiont strains which increase the survival and reproductive output of female hosts. Thus, symbiont strains are favoured which directly increase the survival of the host female through metabolic and physiological contribution to their host. Alternatively, and not exclusively, selection may favour symbiont strains which bias sex allocation towards the production and survival of female offspring. Both symbionts which are 'host-beneficial' (playing a metabolic role) (Douglas, 1989) and symbionts which are 'host-parasitic' (distorting the sex ratio away from the Fisher/Hamilton optimum) are observed. Distortion of the primary sex ratio has been observed in Crustacea (Dunn *et al.*, 1994; Juchault *et al.*, 1994; Juchault & Legrand, 1989). Further to this, microbial induction of parthenogenesis is observed in several species of Hymenoptera (Stouthamer *et al.*, 1990; Zchori-Fein *et al.*, 1994*a*, *b*; Pijls *et al.*, 1996). In non-hymenopteran insects, distortions of the secondary sex ratio are more common, 'male-killing' microorganisms having been recorded in many species (Hurst, 1993).

This study is concerned with the incidence of the different types of symbiosis. A particular bacteria–insect symbiosis can be host-beneficial or host-parasitic. One possibility is that only members of certain bacterial taxa can evolve the different host manipulations found (cytoplasmic incompatibility, parthenogenesis induction, male-killing). The use of

* Corresponding author. Tel: +44(0) 1223 333975. Fax: +44(0) 1223 333992. E-mail: gddh@mole.bio.cam.ac.uk. For offprint requests: Greg Hurst, Department of Biology, Wolfson House, University College London, 4 Stephenson Way, London NW1 2HE, UK.

polymerase chain reaction (PCR) amplification of the 16S rDNA from the bacterial genome has allowed us to resolve the affiliation of microorganisms, irrespective of the ability to culture them *in vitro* (Weisburg *et al*., 1991). We have thus begun to assess the phylogenetic range of bacteria associated with each type of host–symbiont relationship.

So far, parthenogenesis induction and cytoplasmic incompatibility have been observed to be associated with bacteria of one clade: the alpha proteus-like bacterium *Wolbachia* (Stouthamer *et al*., 1993; Van Meer *et al*., 1995; Zchori-Fein *et al*., 1995; Pijls *et al*., 1996). On the other hand, in the three cases where male-killing agents have been identified, bacteria from two different eubacterial groups have been observed: a member of the Gram positives (a *Spiroplasma*) in *Drosophila willistoni* (Hackett *et al*., 1985), and members of the Gram-negative proteobacteria in *Nasonia vitripennis* (bacterium: *Arsenophonus nasoniae*) (Werren *et al*., 1986; Gherna *et al*., 1991) and *Adalia bipunctata* (bacterium: a member of the genus *Rickettsia*) (Werren *et al*., 1994; Hurst *et al*., 1996*a*).

This survey shows that male-killing is a more widely evolved trait than cytoplasmic incompatibility and parthenogenesis induction. It is also tempting to postulate that male-killing should evolve easily, in many bacterial taxa. It is, after all, merely sex-limited lethality. However, paucity of data forbids us from concluding that inherited bacteria from many taxa can evolve male-killing behaviour. Notably within the Gram-negative bacteria, male-killing has not been observed outside the proteobacteria, despite the fact that inherited symbionts exist outside this group (Spirochaetes such as *Borrelia*; *Blattabacterium*, a member of the Flavobacteria–Bacteroides group). Is it that all taxa of inherited bacteria can evolve male-killing, or is the behaviour more restricted in its evolution?

We here identify the male-killing bacterium of the ladybird *Coleomegilla maculata* through 16S rDNA sequence analysis. This result shows male-killing behaviour to have evolved in a Gram-negative bacterium outside the proteobacteria.

2. Materials and methods

Genomic DNA was concomitantly extracted from infected (SR), uninfected (NSR), antibiotic-cured and revertant female *Coleomegilla maculata*, bred in a previous study (revertant females are progeny of females from SR lines which did not display the trait; antibiotic cured females are the F_2 following two generations of treatment with tetracycline; for details of lines see Hurst *et al*., 1996*b*).

Briefly, abdomens were detached, rinsed in sterile water, ground in 250 μl of digestion buffer (80 mM-EDTA, 1% SDS, 160 mM sucrose, 100 mM-Tris-HCl pH 8·0) and incubated overnight at 37 °C in the presence of 20 μg proteinase K. The DNA was purified by shaking with an equal volume of phenol–chloroform (1:1), cleaned with an equal volume of chloroform, ethanol precipitated, washed with 70% ethanol, and resuspended in 100 μl of sterile distilled water.

The 16S rDNA was then amplified by PCR, using as template 1 μl of a 10% solution of the genomic DNA and the primers 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495r (5′-CTACGGCTACCTTGTTACGA-3′), derived from Weisburg *et al*. (1991). Each reaction was of 50 μl total volume containing $1 \times (NH_4)_2SO_4$ buffer (Bioline), 5 mM-$MgCl_2$, 1 unit of *Taq* polymerase (Bioline), 0·2 mM of each nucleotide (Pharmacia), 20 pmol of each primer. Cycle conditions on a Hybaid omnigene machine were: start for 2 min at 94 °C; then 35 cycles of 15 s at 94 °C, 30 s at 55 °C, 3 min at 72 °C; 1 cycle of 15 s at 94 °C, 30 s at 55 °C, 20 min at 72 °C. The resultant PCR product was treated for half an hour with 10 units of T4 polymerase at 37 °C in the presence of nucleotides, run out on a 1% agarose gel against a size standard, ethidium bromide stained, and agarose containing the DNA excised under long-wavelength ultraviolet light.

The DNA was liberated from the gel segment using Gene-clean (Biorad), and ligated overnight at room temperature into *Eco*RV cut pBluescript in the presence of *Eco*RV, ATP and polyethylene glycol. The plasmid was transformed into XL1 Blue *E. coli*, plated using a Blue-White screen, and white colonies picked. These colonies were grown in overnight culture, and the plasmid extracted using alkaline lysis. The size of insert borne by this plasmid was then checked by restriction digestion. Two clones containing inserts of the appropriate size were obtained from each of two individuals from different sex ratio lines (normal lines and no template controls gave no PCR product). These were then sequenced using cycle sequencing in the presence of dye-labelled terminators, with the resultant products being visualized on an ABi automated sequencer. Primers internal to the insert were designed and used in sequencing, to ensure full coverage of both strands.

The four sequences obtained were identical within the error rates expected from *Taq* polymerase (mean 2 or 3 differences over 1454 bases), and this sequence taken putatively as representing the male-killing bacterium.

This hypothesis was tested in two ways. The sequence obtained was compared with a wide variety of eubacterial sequences in the EMBL and Genbank databases, and unusual restriction sites sought. Following this, PCR amplification products from each of the six infected lines were tested for the presence of an *Nru*I site 720 bases into the sequence. Thus, 9 μl of PCR-product-derived amplification of infected and uninfected template was digested for 2 h at 37 °C in a 25 μl total volume, containing appropriate buffer and

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10 units of restriction enzyme *Nru*I. The products were then run out on a gel alongside undigested product.

Further to this, oligonucleotide primers with 3′ ends specific to the putative male-killer were designed for use in PCR. The sequence of these primers was 5′-ATTGTTAAAGTTCCGGCG-3′ (forward) and 5′-CTGTTTCCAGCTTATTCGTAGTAC-3′ (reverse). These correspond to *E. coli* 16S rDNA positions 202–220 and 1022–997, respectively. The association with the putative microorganism was then tested by PCR. The expectation was of a product 762 bp in length in samples bearing the bacterium, with no product from other samples. One microlitre of a 10% solution of genomic DNA derived from infected females from each of six lines, from uninfected individuals from each of five lines, one 'natural revertant' individual from each of three previously infected lines, and one antibiotic-cured individual from each of the three remaining lines, was then subjected to PCR, using the PCR mix described above, with the two new primers. The cycling conditions were: 1 cycle at 94 °C for 2 min, 60 °C for 1 min, 72 °C for 1 min 30 s; 35 cycles at 94 °C for 15 s, 60 °C for 1 min, 72 °C for 1 min 30 s; 1 cycle at 94 °C for 15 s, 59 °C for 1 min, 72 °C for 10 min. Ten microlitres of product was run out on a 1% agarose gel, against a size standard, and stained with ethidium bromide. Any amplified DNA was excised from the gel, cleaned, and partially directly sequenced using the reverse PCR primer to ensure it represented the expected product in sequence as well as length. The specificity of the reaction was tested by attempting to amplify a range of eubacterial DNA with the primers, using the same conditions (*Bacillus cereus*; *E. coli*; *Flavobacterium aquatile*; *Pseudomonas syringii*; *Streptococcus faecalis*; *Staphylococcus aureus*; *Rickettsia*-infected *A. bipunctata*, *Spiroplasma*-infected *A. bipunctata*).

As a control against poor quality of genomic DNA causing amplification failure, the quality of the insect genomic DNA extractions that failed to amplify in the specific reaction was verified by attempting amplification with a pair of general primers which amplifies a portion of the beetle *COI* gene of mtDNA (Howland & Hewitt, 1995). The quality of bacterial genomic DNA was tested using the general bacterial amplification above.

The sequence obtained for the male-killing bacterium of *C. maculata* was manually aligned with groups of prealigned 16S rDNA sequences representing the main eubacterial lineages (Maidak *et al.*, 1994), the five main groups of the Flavobacteria–Bacteroides lineage (Gherna & Woese, 1992), and the genus *Blattabacterium* (Bandi *et al.*, 1994, 1995). Phylogenetic analysis was effected using TREECON (Van de Peer & De Wachter, 1993) according to distance-matrix methods (Kimura's correction, Neighbour Joining method) after both including or excluding the 16S rDNA variable regions and any insertions/deletions; the robustness of the result was evaluated by bootstrap analysis.

3. Results

The four clones from the two individuals bore the same sequence, 1454 bp in length, save for 2 or 3 bases per sequence which may be regarded as cloning errors (sequence submitted to EMBL, ref. y13889). This sequence contained the sequence motif recognized by the six-cutting enzyme *Nru*I, which cleaves the sequence into one fragment of 720 bases, and another of 770 bases. This site is present in this bacterium, and *Blattabacterium*, but is absent from other bacteria searched in the database. PCR products from each of the infected lines were successfully cut by this enzyme to produce the expected restriction digest pattern; no PCR products were obtained from uninfected individuals (results not shown).

The 'specific' PCR reaction, designed to produce amplifications from the sequence in question, gave products of the expected size when genomic DNA from each of the infected lines was used as template. However, template from individuals from uninfected lines, and template from individuals from sex ratio lines that had reverted to a normal sex ratio naturally, or through antibiotic treatment, did not generate any product (Fig. 1). These templates did produce product with amplifications using conserved primers from the *COI* region of insect mtDNA, indicating the template DNA was of good quality (results not shown). Direct sequencing verified that the products obtained from SR lines were identical in sequence to the putative

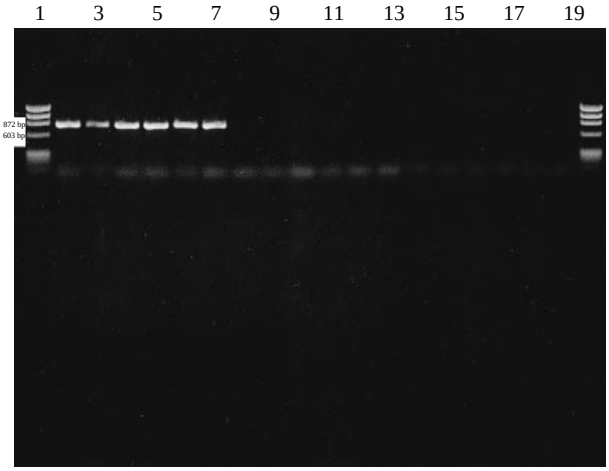

Fig. 1. Amplification products using the *Coleomegilla maculata* putative symbiont-specific PCR reaction, described in the text, with various *C. maculata* templates. Templates: lanes 2–7, female from each of six SR lines; lanes 8–12, female from each of five normal lines; lanes 13–15, 'natural revertant' female from each of three previously SR lines; lanes 16–18, female from each of three SR lines after two generations of curing with antibiotics; lane 19, water control. Lanes 1 and 20 contain ϕ *Hae*III marker.

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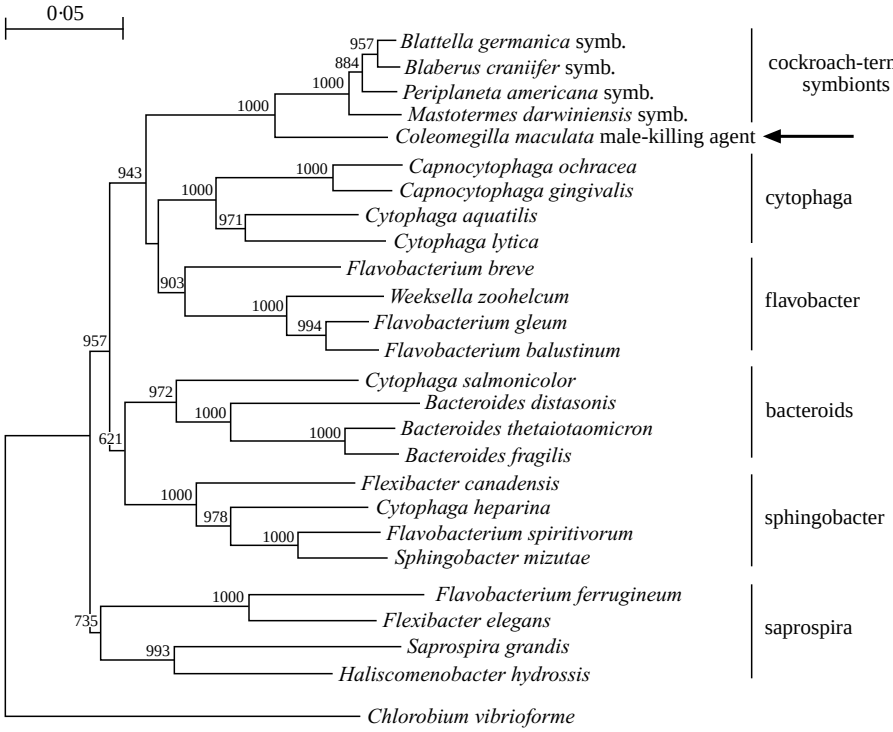

Fig. 2. Phylogenetic tree showing the position of the *C. maculata* symbiont relative to other members of the Flavobacteria/Flexibacter/Cytophaga/Bacteroides group, with the results of 1000 bootstrap replicates. Tree was constructed using Neighbour Joining, distances corrected according to Kimura, from the sequence obtained ignoring variable regions and insertions/deletions.

causal agent: an average of 350 bases were sequenced using the reverse PCR primer above; these were identical (within one base) to the putative 16S rDNA sequence in all six cases.

The 'specific' PCR produced no amplification products with template from other eubacteria, whilst positive controls (infected beetles) did amplify. Again, the quality of the DNA was not the reason for the failure of amplification, amplification being successfully achieved using general primers for 16S rDNA (results not shown). A BLAST search (Altschul *et al.*, 1990) found that the 20 sequences most closely matching that of our bacterium were members of the Flavobacteria–Bacteroides group. This result was corroborated by sequence signature analysis. Woese *et al.* (1990) have delineated ten sequence features of Flavobacteria–Bacteroides 16S rDNA that are characteristic of this group but are either very rarely or never found in the sequence of the 16S rDNA in other groups. Within the section of 16S rDNA that we sequenced, there were nine potential signature features. In each case, the sequence was the same as that expected for a member of the Flavobacteria–Bacteroides group.

We constructed a phylogenetic tree to delineate the precise position of our bacterium within the Flavobacteria–Bacteroides group. Excluding variable regions and insertions and deletions, this tree suggested the male-killing bacterium in *C. maculata* to be the sister lineage to *Blattabacterium*, the beneficial symbiont of cockroaches and termites. This conclusion was reinforced by the high level of bootstrap support for the *Blattabacterium–C. maculata* symbiont node (1000 of 1000 replicates) (Fig. 2). The robustness of this node is further borne out by its presence in trees constructed including insertions and deletions, trees constructed when variable sections of the sequence were included, and trees in which all flavobacterial sequences were included (results not shown).

The average distance (nucleotide divergence, Kimura corrected) between the *C. maculata* male-killer and the cockroach symbionts is 11·49%, compared with an average difference of 6·3% between the cockroach symbionts and the termite symbiont. This latter divergence being around 200 Ma BP (Bandi *et al.*, 1995), we can infer that the *C. maculata* symbiont split from the cockroach and termite endosymbionts around the Palaeozoic (*c.* 400 Ma BP). This date is compatible with that obtained through direct use of the 16S rDNA clock calibrated by Moran *et al.* (1993) (divergence 1–2% per 50 Ma: 5·75% divergence per lineage equates to 280–575 Ma).

4. Discussion

This paper concerns one principal question: why are not all inherited symbionts male-killers, inducers of cytoplasmic incompatibility or inducers of host parthenogenesis? Previous studies have suggested that the induction of cytoplasmic incompatibility is a product of one clade of bacteria: *Wolbachia* (Breeuwer *et al.*, 1992; O'Neill *et al.*, 1992; Hoshizaki & Shimada,

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1995). This has been interpreted as being the product of the difficulty of the host manipulation involved (Hurst 1993). Cytoplasmic incompatibility is subtle: the condensation of the paternal chromosome set of an uninfected female through (it is considered) products placed in sperm by infected males (Breeuwer & Werren, 1990). Parthogenesis induction is similarly associated with this bacterial clade (Stouthamer *et al.*, 1993; Zchori-Fein *et al.*, 1995; Pijls *et al.*, 1996). It is similarly subtle, this time involving a manipulation of the early mitotic division in haploid (male) individuals (Stouthamer & Kazmer, 1994).

This study, on the other hand, supports the belief that male-killing is a widely evolved trait. Four studies have shown bacteria from three different groups (Mollicutes, Proteobacteria and now a member of the Flavobacteria–Bacteroides group) to have evolved male-killing behaviour. This diversity is consistent with the notion that male-killing is relatively easy to evolve. Our data point thus reinforces the view that there is no 'systematic' bar to the evolution of male-killing behaviour, and we suggest that transovariolly transmitted bacteria from any bacterial taxon could evolve this behaviour.

We therefore suggest that non-*Wolbachia* bacteria that are strictly vertically transmitted evolve to be either host-beneficial (through providing a nutritional role) or host-parasitic (the death of males is a large cost, and we therefore consider it highly unlikely that male-killers will be anything other than parasitic). This is stated notwithstanding the possibility that a male-killer could have positive direct effects on the female host (through a nutritional role), as such direct effects are not likely to compensate the female fully for the loss of her male progeny. We suspect that the nature of the relationship that evolves (host-parasitic, host-beneficial) will depend upon host-specific factors. Two host factors are possible. First, we could imagine that certain hosts are not susceptible to male-killing behaviour, by virtue of differences in physiology or systems of sex determination. However, the taxonomic breadth of the hosts of male-killing bacteria suggests that this is not the case. Male-killing bacteria are observed in a wide variety of insect orders (Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera) (Hurst, 1991). The second and most important factor is host ecology. This will dictate whether killing male embryos is advantageous to the symbiont. Male-killing is only advantageous in hosts where there are either antagonistic interactions between siblings (e.g. competition for food, cannibalism), deleterious inbreeding between siblings, or sibling egg consumption (Skinner, 1985; Werren, 1987; Hurst, 1991). Particular insect groups, by virtue of their ecology, are more likely to possess male-killers than others. Coccinellid beetles, for instance, generally exhibit sibling egg cannibalism, where neonate larvae eat eggs that fail to hatch. This ecology is perfect for the spread of male-killers (Hurst & Majerus, 1993), and indeed five incidences of male-killing have been recorded in this group (for review see Hurst *et al.*, 1997).

We thus predict that male-killing will have evolved in a wide range of bacterial taxa in hosts with suitable ecology, and that beneficial symbioses will therefore be most likely to evolve in hosts where the ecology is not permissive to the spread of a mutant symbiont which kills male hosts.

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