

Endosymbiotic Bacteria of Bed Bugs: Evolution, Ecology and Genetics

Joyce M. Sakamoto and Jason L. Rasgon

The human bed bug (*Cimex lectularius* L.) (Fig. 1) is an ancient associate of human beings (Panagiotakopulu and Buckland 1999). In developed countries, bed bugs have not been serious household pests since World War II because of DDT applications and improved sanitary standards (Usinger 1966, Temu et al. 1999, Gangloff-Kaufmann and Shultz 2003). Within the past 20 years, however, there has been a drastic increase in the number of reported bed bug infestations in developed countries (King et al. 1989, Krueger 2000, Boase 2001). This increased incidence has been attributed to second-hand goods and furniture, increased international traffic, and growing pesticide resistance (King et al. 1989, Boase 2001), but the ultimate causes are still under some debate.

The resurgence of bed bug outbreaks has stimulated interest in the development of novel control strategies for this pest. Current chemical control methods include application of pyrethroids, insect juvenile hormone analogues, and organophosphates (Boase 2001, Jacobs 2003). Rapid development of resistance to chemical agents has been observed in natural bed bug populations. DDT resistance was first reported in 1947 and has been observed in *C. lectularius* and the related species *C. hemipterus* (F.) (Usinger 1966, Busvine 1971). In the 1970s, organophosphate resistance was observed (Feroz 1971). Pyrethrin tolerance as a consequence of DDT cross-resistance also was reported (Busvine 1971).

Bed bug resistance to or tolerance of pyrethrins and pyrethroids is of additional public health importance because a major incentive for using pyrethroid-treated bed nets for control of malaria-transmitting mosquitoes is bed bug control (WHO 1997, Temu et al. 1999). Resistant bed bugs have contributed to a loss of motivation among villagers to continue treating bed nets, which has resulted in increased risk for contracting malaria (WHO 1997, Myamba et al. 2002).

Molecular control methods have been proposed in other insect systems of medical, veterinary, and agricultural importance. One potential novel method is to manipulate obligate endosymbiotic bacteria (Beard et al. 1998). The idea is that by manipulating or eliminating symbionts required by the arthropod for blood meal digestion, reproduction, or develop-

ment, arthropods can be sterilized, killed, or have their life cycles disrupted, which will result in the reduction or elimination of populations. These strategies have the potential to be efficacious and cost-effective. In principle, endosymbionts can be targeted specifically, and thus these strategies should be safe and have minimal nontarget effects.

Endosymbionts were first documented by light microscopy in bed bugs almost 90 years ago (Arkwright et al. 1921, Usinger 1966, Chang and Musgrave 1973). More recent molecular studies identified two symbionts in *C. lectularius* that belong to two distinct bacterial groups. The first symbiont was identified as *Wolbachia* (Hypsa and Aksoy 1997, Rasgon and Scott 2004, Sakamoto and Rasgon 2006, Sakamoto et al. 2006), an alpha-proteobacterium that is a common invertebrate endosymbiont (Werren et al. 1995, Werren 1997, Jeyaprasak and Hoy 2000 (Fig. 2). The second endosymbiont was related to an unnamed gamma-proteobacterial symbiont in the planthopper *Euscelidius variegatus* (Hypsa and Aksoy 1997; JLR, unpublished data) and was termed BEV-like symbiont (Bacteria of *E. variegatus*) (Campbell and Purcell 1993).

Speculations have arisen about the role that individual endosymbionts may play in bed bug biology. Some have suggested that bed bugs might obtain B vitamins from at least one of their endosymbionts (De Meillon and Goldberg 1947). Severe reductions in egg production have been observed in bed bugs when symbionts were eliminated by heat (Chang 1974) or antibiotic treatment (Takano-Lee et al. 2003). Because bed bugs seem to be dependent on one or more of their symbionts, these bacteria may serve as a novel, specific target for control efforts. Along these lines, we have been investigating the distribution of endosymbionts across the family Cimicidae in general and in more detail within the human bed bug *C. lectularius*.



Fig. 1. Engorged female *Cimex lectularius*, the human bed bug.

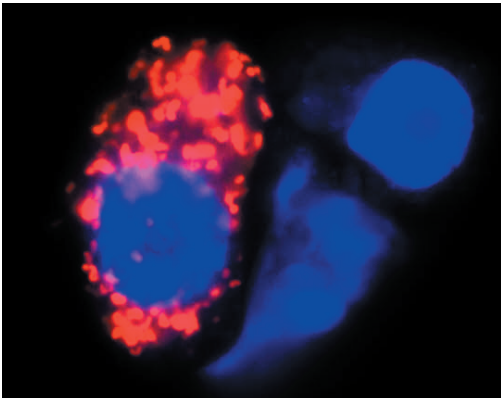


Fig. 2. *Wolbachia* visualized in insect cells by fluorescence *in situ* hybridization using *Wolbachia*-specific oligonucleotide probes. Red, *Wolbachia*. Blue, host cell nuclei stained with DAPI.

Wolbachia

Wolbachia has been identified infecting a diverse range of invertebrates (Werren et al. 1995, Hypsa and Aksoy 1997, Stouthamer et al. 1999, Vandekerckhove et al. 1999, Werren and Windsor 2000, Lo et al. 2002, Rasgon and Scott 2004, Rowley et al. 2004, Bordenstein and Rosengaus 2005). *Wolbachia* is estimated to infect up to 70% of all known insect species, making it perhaps the most prevalent symbiotic bacterium on the planet (Jeyaprakash and Hoy 2000). Eight major *Wolbachia* phylogenetic “supergroups” (A–H) are currently recognized (Fig. 3). A, B, and

E infect diverse arthropods; C and D infect nematodes; G infects spiders; H infects termites; and F infects arthropods and nematodes (Stouthamer et al. 1999, Vandekerckhove et al. 1999, Lo et al. 2002, Rasgon and Scott 2004, Rowley et al. 2004, Bordenstein and Rosengaus 2005). Infection with *Wolbachia* is commonly associated with host reproductive phenotypes, such as cytoplasmic incompatibility (sterility when infected males mate with uninfected females or females infected with a different *Wolbachia* strain), feminization, male-killing, parthenogenesis, increased or decreased fitness, and obligate symbiosis (Stouthamer et al. 1999).

Wolbachia-like inclusions were identified in bed bugs by microscopy almost 90 years ago (Arkwright 1921). DNA sequencing of bacterial 16S rDNA conclusively identified F supergroup *Wolbachia* in a laboratory colony of *C. lectularius* (Hypsa and Aksoy 1997, Rasgon and Scott 2004). More recently, we investigated the geographic distribution of *Wolbachia* infections in natural *C. lectularius* populations in North America and Africa. *Wolbachia* infections were found at high prevalence (83–100%) in all sampled populations

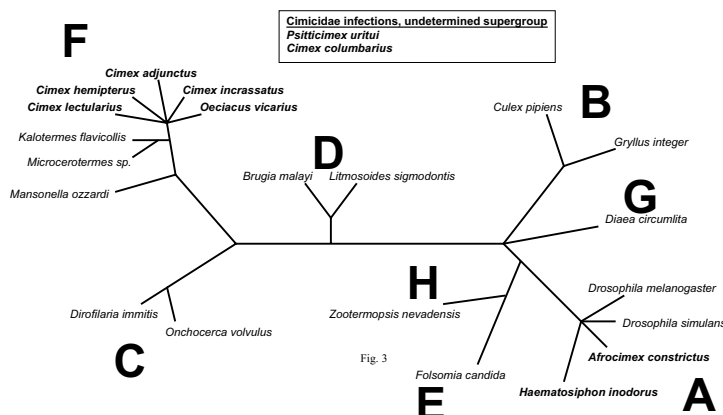


Fig. 3. Unrooted dendrogram of *Wolbachia* 16S rDNA gene sequences, based on previously published sequence data (Lo et al. 2002, Rowley et al. 2004, Bordenstein and Rosengaus 2005, Sakamoto et al. 2006). Bold indicates cimicid infections. Taxon names represent host species. Letters indicate *Wolbachia* supergroup designations.

(Sakamoto and Rasgon 2006). Because targeting an influential bacterium for control strategies will not be useful if the endosymbiont is not ubiquitous, high infection levels indicate that *Wolbachia* may be useful as a novel potential target for control efforts.

The specific effect of *Wolbachia* on bed bug biology is unknown. CI-like crossing patterns were observed during experimental crosses between *C. lectularius* and the closely related species *C. columbarius* (sometimes considered a subspecies). When *C. lectularius* males mated with *C. columbarius* females, the number of oviposited eggs decreased significantly, whereas the reciprocal cross was fertile (Ueshima 1964). We have identified *Wolbachia* infections in *C. columbarius* and *C. lectularius* (Sakamoto et al. 2006), but it likely that these two infections are distinct from one another.

Historically, *Wolbachia*-like inclusions were observed in other cimicids besides *C. lectularius* and were identified by PCR (polymerase chain reaction) in the cliff swallow bug *Oeciacus vicarius* Horvath (Rasgon and Scott 2004). We therefore wondered how common *Wolbachia* infections were within the family Cimicidae in general. We used PCR to screen for *Wolbachia* infections in multiple cimicid species. These experiments were complicated by the fact that apart from species of medical importance, cimicids that feed on nonhuman hosts are not frequently collected. Museum specimens were previously used for molecular surveys of other bacteria (Persing et al. 1990, Barns et al. 2000), and we speculated that preserved museum material could be used in a similar manner for *Wolbachia* surveys. Generous donations of specimens from private donors, and in particular from the Usinger Cimicid Collection housed at the Essig Museum of Entomology (University of California, Berkeley), allowed us to obtain a broad sampling across the family for screening purposes. Nondestructive DNA extraction methods were used to preserve valuable specimens for future study (Sakamoto et al. 2006).

We screened 39 cimicid species for *Wolbachia* infection (spanning 16 genera and all 6 recognized subfamilies) (Usinger 1966), using primers specific to the *Wolbachia* 16S rDNA gene. PCR was initially attempted using published primers (O’Neill et al. 1992, Werren and Windsor 2000). Amplification of PCR products >500 bp (base pairs) generally succeeded from recently collected wild specimens (*Afrocimex constrictus* Ferris and Usinger and *C. lectularius*), but usually failed when attempted on preserved museum material due to the degraded nature of the template DNA. We therefore designed primers to amplify small overlapping fragments (130–240 bp) of the *Wolbachia* 16S rDNA gene. From degraded specimens, amplification success rate of small fragments (<200 bp) was much greater than larger fragments. These sequences were concatenated for phylogenetic analysis. Using this technique, we were able to detect and sequence *Wolbachia*-specific DNA fragments in preserved

cimicid specimens over 40 years old and use this information to place most infection into a phylogenetic context (Sakamoto et al. 2006).

Even though DNA in most specimens was highly degraded, we were able to detect, and confirm by sequencing, *Wolbachia* DNA in 23% of cimicid species (9/39). We identified nine cimicid infections, seven of which were previously undescribed. Infections in the F supergroup were common in the subfamily Cimicinae (*C. lectularius*, *C. hemipterus*, *C. adjunctus* Barber, *C. incrassatus* Usinger and Ueshima, and *Oeciacus vicarius*). Infections in the A supergroup were identified in the subfamilies Afrocimicinae (*Afrocimex constrictus*) and Haematosiphoninae (*Haematosiphon inodorus* Duges). We were able to detect and confirm by sequencing diagnostic *Wolbachia* fragments from the cimicids *C. columbarius* and *Psitticimex uritui* (Lane and Abalos), but we did not obtain enough sequence information to phylogenetically place these infections into a supergroup (Fig. 3) (Sakamoto et al. 2006).

The observation of multiple F supergroup infections among the subfamily Cimicinae is very interesting (Fig. 3). These F infections were observed in two genera (*Cimex* and *Oeciacus*) and appear to be monophyletic, which suggests that *Wolbachia* was introduced once and has diverged dependently along with the insect hosts in this subfamily. In contrast, A supergroup infections were detected in two widely divergent subfamilies (Afrocimicinae and Haematosiphoninae), suggesting multiple introductions of A infections into bed bugs.

Our initial estimates of *Wolbachia* prevalence in cimicids are almost certainly an underestimate. Failure to detect *Wolbachia* DNA in many species may be due to lack of infection, sampling bias, or most likely, poor template quality in insufficiently preserved museum specimens. Many museum specimens in our study were stored without temperature control or ethanol changes for more than 40 years. Even with poorly preserved material, we observed an infection rate in cimicids of (23%) comparable to other estimates of *Wolbachia* prevalence in arthropod taxa (Werren and Windsor 2000). Our results thus suggest that *Wolbachia* infections are probably common among bed bugs in general.

BEV-like Symbiont

The second characterized symbiont known to infect *C. lectularius* is the BEV-like symbiont (BLS). BLS was identified by sequencing specific fragments of bacterial 16S rDNA from a laboratory colony (NIH) of *C. lectularius* (Hypsa and Aksoy 1997). We have confirmed this result in a different bed bug colony (Maryland) and have detected the symbiont in several wild specimens from Africa (JLR and JMS, unpublished). The geographical distribution of BLS and infection frequencies in natural populations, however, is currently unknown.

Endosymbiotic bacteria related to BLS have been identified by DNA sequencing in several insect taxa, including ant lions (Dunn and Stabb 2005) and tick flies (Reeves 2005), in addition to

E. variegatus and cimicids, and thus this group may reflect a previously undescribed clade of widespread insect endosymbiotic bacteria. The phenotypic effect of BLS on bed bug biology is unknown. When bugs were fed on blood supplemented with penicillin–streptomycin (pen–strep), reductions in egg production were observed. *Wolbachia* is not susceptible to pen–strep (Fenollar et al. 2003), which suggests the possibility that BLS is required for egg development in bed bugs. However, the possibility of another undescribed symbiont in bed bugs that is required for reproduction cannot be ruled out at this time. We are currently conducting experiments to characterize the interaction between BLS and bed bugs at the phenotypic and genetic level to address this question.

Acknowledgments

We thank the following people for providing specimens used in our studies: Nixon Wilson, Carl Dick, Klaus Reinhardt, Cheryl Barr, Douglas Norris, Rebekah Kent, Neeta Pardanani, Joseph E. Kuntz, Fred Rozo, Laura Harrington, Becky Poulson, William H. Kern, Peggy Nusser, Lynn Frank, Stephen W. Hwang, Susan Hacker, John Mangold, and Robin Todd. Special thanks to Harold Harlan for insights into new outbreaks and live specimens, and to Brian Cabrera and Kathy Heinsohn for organizing the symposium at the 2005 ESA Annual Meeting. We thank John Werren for helpful comments and Julie Feinstein for assistance with primer design. Funding for much of this research was provided by the Johns Hopkins Malaria Research Institute and NSF FIBR grant EF-0328363.

References

- Arkwright, J. A., E. E. Atkin and A. Bacot. 1921. An hereditary *Rickettsia*-like parasite of the bed bug (*Cimex lectularius*). *Parasitology* 13: 27–36.
- Barns I., J. Holton, D. Vaira, M. Spigelman and M. G. Thomas. 2000. An assessment of the long-term preservation of the DNA of a bacterial pathogen in ethanol-preserved archival material. *J. Pathol.* 192: 554–559.
- Beard C. B., R. V. Durvasula and F. F. Richards. 1998. Bacterial symbiosis in arthropods and the control of disease transmission. *Emerg. Infect. Dis.* 4: 581–591.
- Boase, C. 2001. Back from the brink. *Pesticide Outlook* 12: 159–162.
- Bordenstein S. and R. B. Rosengaus. 2005. Discovery of a novel *Wolbachia* supergroup in isoptera. *Curr. Microbiol.* 51: 393–398.
- Busvine, J. R. 1971. Cross-resistance in arthropods of public health importance, parts I and II. WHO/VBC/71.307.
- Campbell, B. C. and A. H. Purcell. 1993. Phylogenetic affiliation of BEV, a bacterial parasite of the leafhopper *Euscelidius variegatus*, on the basis of 16S rDNA sequences. *Curr. Microbiol.* 26: 37–41.
- Chang, K. P. 1974. Effects of elevated temperature on the mycetome and symbiotes of the bed bug *Cimex lectularius* (Heteroptera). *J. Invertebr. Pathol.* 23: 333–40.

- Chang, K. P., and A. J. Musgrave. 1973. Morphology, histochemistry, and ultrastructure of mycetome and its rickettsial symbiotes in *Cimex lectularius* L. Can. J. Microbiol. 19: 1075–1081.
- De Meillon, B., and L. Golberg. 1947. Preliminary studies on the nutritional requirements of the bedbug (*Cimex lectularius* L.) and the tick *Ornithodoros moubata* Murray. J. Exp. Biol. 24: 41–63.
- Dunn, A. K., and D V Stabb. 2005. Culture-independent characterization of the microbiota of the ant lion *Myrmeleon mobilis* (Neuroptera: Myrmeleontidae). Appl. Environ. Microbiol. 71: 8784–8794.
- Fenollar F., M. Maurin and D. Raoult. 2003. *Wolbachia pipientis* growth kinetics and susceptibilities to 13 antibiotics determined by immunofluorescence staining and real-time PCR. Antimicrob. Agents Chemother. 47: 1665–1671.
- Feroz, M. 1971. Biochemistry of malathion resistance in a strain of *C. lectularius* resistant to organophosphorus compounds. Bull. WHO 45: 795–804.
- Gangloff-Kaufmann, J., and J. Shultz. 2003. Bed bugs are back! An IPM answer. New York State Integrated Pest Management Program leaflet. Cornell Cooperative Extension. Retrieved on 12 Sept. 2005 at www.nysipm.cornell.edu
- Hypsa, V., and S. Aksoy. 1997. Phylogenetic characterization of two transovarially transmitted endosymbionts of the bedbug *Cimex lectularius* (Heteroptera: Cimicidae). Insect Mol. Biol. 6: 301–304.
- Jacobs, S. B. 2003. Bed bugs: *Cimex lectularius*. Entomological Notes, Pennsylvania State College of Agricultural Sciences Cooperative Extension, Department of Entomology. Retrieved on 13 Dec. 2004 at: http://www.ento.psu.edu/factsheet/bed_bug.htm
- Jeyaprakash, A., and M. A. Hoy. 2000. Long PCR improves *Wolbachia* DNA amplification: *usp* sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. 9: 393–405.
- King E., I. Dick, and P. Evans. 1989. Bed bugs in Britain. Parasitol. Today 5: 100–102.
- Krueger, L. 2000. Don't get bitten by the resurgence of bed bugs. Pest Control 68: 58–64.
- Lo N, M. Casiraghi, E. Salati, C. Bazzocchi, and C. Bandi. 2002. How many *Wolbachia* supergroups exist? Mol. Biol. Evol. 19: 341–346.
- Myamba, J., C. A. Maxwell, A. Asidi, and C. F. Curtis. 2002. Pyrethroid resistance in tropical bedbugs, *Cimex hemipterus*, associated with use of treated bednets. Med. Vet. Entomol. 16: 448–451.
- O'Neill S L, R. Giordano, A. M. E. Colbert, T. L. Karr and H. M. Robertson. 1992. 16 S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA 89: 2699–2702.
- Panagiotakopulu, E., and P. C. Buckland. 1999. *Cimex lectularius* L., the common bed bug from Pharaonic Egypt. Antiquity 73: 908–911.
- Persing D. H., S. R. Telford III, P. N. Rys, D. E. Dodge, T. J. White, S. E. Malawista, and A. Spielman. 1990. Detection of *Borrelia burgdorferi* DNA in museum specimens of *Ixodes dammini* ticks. Science 249: 1420–1423.
- Rasgon, J. L., and T. W. Scott. 2004. Phylogenetic characterization of *Wolbachia* symbionts infecting *Cimex lectularius* L. and *Oeciacus vicarius* Horvath (Hemiptera: Cimicidae). J. Med. Entomol. 41: 1175–1178.
- Reeves, W. K. 2005. Molecular genetic evidence for a novel bacterial endosymbiont of *Icosta americana* (Diptera: Hippoboscidae). Entomol. News 116: 263–265.
- Rowley S. M., R. J. Raven and E. A. McGraw. 2004. *Wolbachia pipientis* in Australian spiders. Curr. Microbiol. 49: 208–214.
- Sakamoto, J. M., and J. L. Rasgon. 2006. Geographic distribution of *Wolbachia* infections in the human bed bug *Cimex lectularius* L. (Heteroptera: Cimicidae). J. Med. Entomol. In press.
- Sakamoto J.M., J. Feinstein and J.L. Rasgon. 2006. *Wolbachia* infections in the Cimicidae: Museum specimens as an untapped resource for endosymbiont surveys. Appl. Environ. Microbiol. 72:3161-3167.
- Stouthamer, R., J. A. J. Breeuwer and G. D. D. Hurst. 1999. *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53: 71-102.
- Takano-Lee, M., R. K. Velten, J. D. Edman, B. A. Mullens, and J. M. Clark. 2003. An automated feeding apparatus for in vitro maintenance of the human head louse, *Pediculus capitis* (Anoplura: Pediculidae). J. Med. Entomol. 40: 795–799.
- Temu, E. A., J. N. Minjas, C. J. Shiff, and A. Majala. 1999. Bedbug control by permethrin-impregnated bednets in Tanzania. Med. Vet. Entomol. 13: 457–459.
- Ueshima, N. 1964. Experiments on reproductive isolation in *Cimex lectularius* and *Cimex columbarius*. Pan-Pac Entomol 40: 47–53.
- Usinger, R. L. 1966. Monograph of Cimicidae: Hemiptera-Heteroptera. Thomas Say Foundation, vol. 7. Entomological Society of America, College Park, MD.
- Vandekerckhove, T. T., S. Watteyne, A. Willems, J. G. Swings, J. Mertens, and M. Gillis. 1999. Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. FEMS Microbiol. Lett. 180: 279–286.
- Werren, J. H. 1997. Biology of *Wolbachia*. Annu. Rev. Entomol. 42: 587–609.
- Werren, J. H. and D. M. Windsor. 2000. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proc. Biol. Sci. London Ser. B 267: 1277–85.
- Werren, J. H., W. Zhang, and L. R. Guo. 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc. Biol. Sci 261: 55–63.
- WHO (World Health Organization). 1997. Chapter 4. Bedbugs, fleas, lice, ticks and mites: Ectoparasites that live on the body, in clothing and in beds, pp. 237–243. In Vector control series, World Health Organization, Geneva, Switzerland.
- Joyce M. Sakamoto, Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore; Jason L. Rasgon, The W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins Malaria Research Institute, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205. e-mail: jrasgon@jhsph.edu.