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The host range of the male-killing symbiont *Arsenophonus nasoniae* in filth fly parasitioids

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ABSTRACT

The Son-killer bacterium, *Arsenophonus nasoniae*, infects *Nasonia vitripennis* (Hymenoptera: Pteromalidae), a parasitic wasp that attacks filth flies. This gammaproteobacterium kills a substantial amount of male embryos produced by an infected female. Aside from male death, the bacterium does not measurably affect the host, and how it is maintained in the host population is unknown. Interestingly, this bacterial symbiont can be transmitted both vertically (from mother to offspring) and horizontally (to unrelated *Nasonia* wasps developing in the same fly host). This latter mode may allow the bacterium to spread throughout the ecological community of filth flies and their parasitoids, and to colonize novel species, as well as permit its long-term persistence.

We tested 11 species of filth flies and 25 species of their associated parasitoids (representing 28 populations from 16 countries) using diagnostic PCR to assess the bacterium's actual host range. In addition to 16S rRNA, two loci were targeted: the housekeeping gene infB, and a sequence with high homology to a DNA polymerase gene from a lysogenic phage previously identified from other insect symbionts. We identified infections of *A. nasoniae* in four species of parasitoids, representing three taxonomic families. Highly similar phage sequences were also identified in three of the four species. These results identify the symbiont as a generalist, rather than a specialist restricted solely to species of *Nasonia*, and also that horizontal transmission may play an important role in its maintenance.

1. Introduction

Insects are commonly infected with microbial symbionts that hinder their host's fitness (i.e., are parasitic), increase their host's fitness (i.e., are beneficial), or exist between these two extremes. Many symbionts are transmitted in a primarily vertical fashion by their insect host, often via the cytoplasm of the egg. This maternal transmission forms a link between the fitness of the host and the symbiont (Werren and O'Neill, 1997). Selection will typically maintain beneficial symbionts within the host population while eliminating more costly symbionts (Hurst, 1991). To this end, symbionts often provide nutrients or protection to their host (Baumann et al., 1995; Montllor et al., 2002; Oliver et al., 2005). Alternatively, some symbionts manipulate their host's reproduction to be maintained in the host population (Stouthamer et al., 1999).

Maternally inherited symbionts are only transmitted by females, causing male hosts to act as an evolutionary dead-end for the symbiont. The symbiont will thus increase its fitness if it causes an infected host to produce more, or higher quality, daughters than an uninfected host. These alterations to the host's reproduction occur by the symbiont either inducing cytoplasmic incompatibility, or by altering the sex-ratio of the offspring produced. Sex-ratio alterations can occur in either the primary sex-ratio, i.e., feminization or induction of parthenogenesis, or in the secondary sex-ratio of the host, i.e., killing males (Werren et al., 2008).

Symbionts that kill males occur in a diverse array of arthropods and can affect several different sex-determination mechanisms (Andreadis, 1985; Hurst, 1991; Hurst and Jiggins, 2000; Nakanishi et al., 2008). Almost all of these agents kill males during the embryonic stage, when it is predicted that (infected) female siblings will benefit the most from male death (Hurst, 1991). These proposed benefits include reduced inbreeding, reduced competition by siblings for local resources, and increased resources through consumption of dead male siblings (Hurst and Jiggins, 2000). Although these benefits are thought to play a crucial role in the maintenance of most male-killers (Hurst et al., 1997), only in one system has a fitness benefit been empirically identified; female pseudoscorpions infected by a male-killing strain of *Wolbachia* produce daughters of both higher quality and number than uninfected females (Koop et al., 2009). How male-killing symbionts are maintained in other systems remains a mystery.

Male-killers that do not benefit their host may rely on horizontal transmission to be maintained. A microsporidium in mosquitoes

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kills males as larvae rather than as embryos (Andreadis, 1985). Upon male death the parasite is released to the environment where it infects additional hosts, and this horizontal transmission is thought to play a crucial role in the maintenance of the parasite (Andreadis, 1985). Although embryonic male-killers are thought to be transmitted primarily maternally, horizontal transmission may augment their successful transmission.

Horizontal transmission may play a critical role in maintaining the male-killing bacterium *Arsenophonus nasoniae* (Gammaproteobacteria: Enterobacteriaceae) in its host *Nasonia vitripennis* (Hymenoptera: Pteromalidae). This wasp is a common pupal parasitoid of flies found in bird nests (e.g. *Protocalliphora sialia*), and an occasional parasitoid of other vertebrate-associated filth flies, for example, those that breed in dung or carrion (Floate et al., 1999; Peters and Abraham, 2010). Also known as Son-killer, *A. nasoniae* inhibits the formation of the maternal centrosome in *N. vitripennis* embryos and causes ~80% of sons to die (Balas et al., 1996; Ferree et al., 2008; Skinner, 1985). The bacterium is found systemically in the bodies of infected females and surviving males without causing apparent effects (either beneficial or detrimental) to the host (Balas et al., 1996; Huger et al., 1985). Wasps infected with *A. nasoniae* have been found across the continental United States with a prevalence of 4–10% (Balas et al., 1996; Skinner, 1985). Within infected lineages, Son-killer is transmitted with high efficiency (95%) from the female to her offspring in an atypical fashion (Skinner, 1985). Symbiont transmission occurs not through the egg, but via the tissue of the wasp's fly host which is mechanically inoculated with bacteria by the female wasp during parasitism (Huger et al., 1985). Wasp larvae acquire Son-killer per-orally and are infected through the midgut (Huger et al., 1985; Skinner, 1985). Interestingly, and perhaps related to this mode of transmission, *A. nasoniae* is unusual for insect symbionts in that it can be easily cultured outside of the host (Gherna et al., 1991; Werren et al., 1986). This ability of the bacterium to live outside cells may allow it to exploit a wide range of hosts.

In the field, multiple female *Nasonia* wasps can parasitize the same fly pupa; i.e., 'superparasitism' (Grillenberger et al., 2008). Under these conditions, all wasp larvae within the fly will interact with the bacteria deposited by an infected female. About 95% of the *Nasonia* emerging from an inoculated host are infected, regardless of their mother's infection status (Skinner, 1985). Many parasitoid wasp species share overlapping host ranges and can co-parasitize a single host (Floate et al., 1999; Floate, 2002). A sympatric congener, *Nasonia longicornis,* is also known to be infected with *A. nasoniae* in the field (Balas et al., 1996). A recent survey also found *A. nasoniae* in *Pachycrepoideus vindemmiae, Muscidifurax raptor*, and *Spalangia cameroni* (Hymenoptera: Pteromalidae), and *Protocalliphora azurea* (Diptera: Calliphoridae) (Duron et al., 2010). This study also showed in experimental wasp co-infections that *A. nasoniae* can be horizontally transferred from *N. vitripennis* to *N. longicornis, Nasonia giraulti,* and *Muscidifurax raptorellus*, and that it causes male-killing in these species (Duron et al., 2010).

In this study we assessed the host distribution of *Arsenophonus* in a community of filth flies and their parasitoid wasps from a wide geographic range. This was done using diagnostic polymerase chain reaction (PCR) and targeting the multi-copy 16S and 23S rDNA loci. A single-copy bacterial gene, infB, has previously been used to resolve the Enterobacteriaceae at a species level (Hedegaard et al., 1999). The infB gene encodes a protein essential for initiating protein synthesis, and here we use this locus to determine the relatedness of *Arsenophonus* strains found in this community. We also screened *Arsenophonus*-positive insects for a phage gene that has been found in a number of related insect symbionts.

2. Methods

2.1. Sample collection

We screened adults from a total of 25 species of Hymenoptera (438 total individuals) and 11 species of Diptera (17 total individuals) for *Arsenophonus* infection. The majority of these samples were used in a previous screening survey for *Wolbachia* (Floate et al., 2006, 2008; Kyei-Poku et al., 2006), and represent individuals collected from both laboratory colonies and the field (Table 1). We also obtained additional insects by collecting mountain bluebird (*Sialia currucoides*) nest material from nest boxes near Lethbridge, AB, on two separate occasions in July, 2008. The nest material was placed within a culture cage incubated at 26 °C (16:8 h light:-dark cycle) and emergent insects were identified using a light microscope.

All insects were individually washed prior to DNA extraction to remove surface contaminants (1 min with 95% EtOH followed by three rinses of sterile dH2O for 1 min each). DNA was extracted using either the Qiagen Blood and Tissue kit, or by the STE method: the insect was ground using a pipette tip in 25 μL of STE buffer (10 mM Tris buffer at pH 8.0, 1 mM EDTA at pH 8.0, 100 mM NaCl) and 5 μL of proteinase K (20 mg/mL), and incubated at 37 °C for 1 h, followed by 3 min at 96 °C. All DNA was stored at −20 °C until use.

We also isolated and cultured *A. nasoniae* from the *N. vitripennis* collected from the bluebird nest material. Under sterile conditions, 26 live wasps were individually washed and decapitated using a razor blade. DNA was extracted from the head of the wasp and used to determine infection status. The wasp body was ground in 50 μL of tryptic soy broth (BACTO) and 30 μL was spread onto GC agar supplemented with Kellogg's supplement B (DIFCO; Kellogg et al., 1963). Plates were incubated at 26 °C for 96 h. Using morphological characteristics (Gherna et al., 1991), *A. nasoniae* colonies were subcultured from infected wasps. Wasps used for bacterial isolation were not included in the survey results. We also used PCR and sequencing to confirm that the cultured isolates were *A. nasoniae* (see below).

2.2. Diagnostic PCR screening

Infection status for *Arsenophonus* was initially assessed by amplifying 23S rDNA from insect samples. For samples displaying a positive band, the 16S locus was subsequently amplified and sequenced. Another locus, infB, was amplified and sequenced from the *A. nasoniae* type strain using degenerate primers. Primers were developed for this locus using Primer3 (Rozen and Skaletsky, 2000) to maximize the mismatches between *A. nasoniae* and closely related bacteria. These novel primers were used to amplify and sequence the infB locus from *Arsenophonus*-positive samples. Finally, during the course of this project we identified a sequence in the type strain of *A. nasoniae* highly similar to the DNA polymerase P45 gene from lysogenic bacteriophage APSE, originally identified in the pea aphid symbiont *Hamiltonella defensa*. It is not known if the sequence we identified is associated with active phage or is stably inherited in *A. nasoniae*. It is also possible that this sequence is associated with symbionts other than *A. nasoniae* (see below). We examined the distribution and evolution of this phage-associated sequence as a window into further understanding the population biology of *A. nasoniae*. As a positive control for our DNA extractions, we amplified a fragment of the low copy insect gene, histone H3, from DNA samples testing negative in all other reactions. PCR conditions and all primer sequences are listed in Table 2.

In all sets of reactions, an appropriate positive control (DNA extracted from *A. nasoniae* from the American Type Culture Collection [ATCC strain 49151], or a previous DNA extraction known to be