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Population dynamics of *Glossina palpalis gambiensis* symbionts, *Sodalis glossinidius*, and *Wigglesworthia glossinidia*, throughout host-fly development

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ABSTRACT

The tsetse fly (Diptera: Glossinidae), the vector of trypanosomes causing human and animal trypanosomiasis, harbors symbiotic microorganisms including the primary symbiont *Wigglesworthia glossinidia*, involved in the fly's nutrition and fertility, and the secondary symbiont *Sodalis glossinidius*, involved in the trypanosome establishment in the fly's midgut. Both symbionts are maternally transmitted to the intrauterine progeny through the fly's milk gland secretions. In this study, we investigated the population dynamics of these symbionts during fly development. *Wigglesworthia* and *Sodalis* densities were estimated using quantitative PCR performed on *Glossina palpalis gambiensis* at different developmental stages. The results showed that the density of the primary *Wigglesworthia* symbiont was higher than that of *Sodalis* for all host developmental stages. *Sodalis* densities remained constant in pupae, but increased significantly in adult flies. The opposite situation was observed for *Wigglesworthia*, whose density increased in pupae and remained constant during the female adult stage. Moreover, *Wigglesworthia* density increased significantly during the transition from the pupal to the teneral stage, while mating had a contradictory effect depending on the age of the fly. Finally, tsetse fly colonization by both symbionts appears as a continuous and adaptive process throughout the insect's development. Last, the study demonstrated both symbionts of *G. p. gambiensis*, the vector of the chronic form of human African trypanosomiasis, to be permanent inhabitants of the colony flies throughout their life span. This was expected for the primary symbiont, *Wigglesworthia*, but not necessarily for the secondary symbiont, *S. glossinidius*, whose permanent presence is not required for the fly's survival. This result is of importance as *Sodalis* could be involved in the tsetse fly vector competence and may constitute a target in the frame of sleeping sickness fighting strategies.

1. Introduction

Tsetse flies are medically and agriculturally important vectors that transmit African trypanosomes, the causative agents of sleeping sickness in humans and nagana in animals. Human African trypanosomiasis (HAT) affects a wide range of Sub-Saharan African populations (WHO, 2006) and is fatal if untreated. Nagana is estimated to cost African agriculture US$4.5 billion per year (Reinhardt, 2002).

In 2000, the number of infected people was estimated at 300,000. In 2009, and for the first time since the 1960s, the number of declared cases was below 10,000 (Simarro et al., 2011); however, because of problems establishing sound disease diagnosis and performing valuable epidemiological surveys in many bush areas and villages, the estimated number of cases was three times higher.

The biological process leading to transmission of the trypanosomes from one mammalian host to another is complex. Prior to being transmitted, the parasite, taken in the infective blood meal, must first establish in the tsetse fly midgut and then mature in the salivary glands or in the mouthparts, depending on the trypanosome species (Vickerman et al., 1988; Van den Abbeele et al., 1999). The vector's ability to acquire the parasite, favor its maturation, and transmit it to a mammalian host is known as vector competence, which depends on both *Glossina* and trypanosome species. The factors involved in trypanosome establishment in the tsetse fly midgut are still largely unknown, and only a small proportion of flies develop mature infection and transmit the parasite (Maudlin and Welburn, 1994). However, *Sodalis glossinidius*, one of the symbionts harbored by the tsetse fly, has been suspected of being involved in the trypanosome establishment process (Maudlin and Ellis, 1985; Welburn et al., 1993). Epidemiological

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investigations conducted in several HAT foci in Cameroon have demonstrated the association between the presence of the symbiont and the establishment of trypanosome species and subspecies in the flies' midgut (Farikou et al., 2010).

The tsetse fly harbors three symbionts including the above-mentioned secondary symbiont, *S. glossinidius* (Aksoy, 1995; Cheng and Aksoy, 1999; Dale and Maudlin, 1999) and the obligate primary symbiont, *Wigglesworthia glossinidia* (Aksoy, 1995; Aksoy et al., 1995). Both are members of the *Enterobacteriaceae* family, are inhabitants of the fly's gut, and are vertically transmitted to the intrauterine-developing larvae (Cheng and Aksoy, 1999).

Wigglesworthia lives within specialized epithelial cells, the bacteriocytes, which form the bacteriome organ in the anterior midgut. Phylogenetic studies have shown that *Wigglesworthia* displays concordant evolution with its host species: its association with the tsetse is ancient, 50–80 million years old (Chen et al., 1999). Its genome, approximately 700 kb in size, displays a streamlined functional capacity. Despite this rather small genome, it encodes a plethora of vitamins and biosynthetic products that may promote host reproduction as well as fly nutrition throughout its development (Akman et al., 2002; Rio et al., 2012). The presence of *Wigglesworthia* is essential for the flies' fecundity. Its absence has been shown to render females sterile. In the absence of the symbiont, vitamin supplementation of the blood meal partially restores host fertility (Nogge, 1976, 1982), indicating that *Wigglesworthia* may have additional functional roles that may not be substituted with only vitamin supplementation.

The secondary symbiont, *Sodalis*, resides in midgut tissues and in other tissues such as muscle, body fat, hemolymph, milk glands, and salivary glands of certain *Glossina* species (Cheng and Aksoy, 1999). The role of *Sodalis* symbiosis in tsetse is relatively unknown, although its specific elimination has been reported to result in a reduction of host longevity (Dale and Welburn, 2001). In addition, it has been implicated in the susceptibility of tsetse for trypanosome transmission (Welburn and Maudlin, 1992; Farikou et al., 2010).

The tsetse larva receives nutrients along with both gut symbionts from its mother via milk gland secretions (Aksoy et al., 1997), during its intrauterine life.

In the context of potential vector control using tsetse fly symbionts, we investigated the population dynamics of these symbionts during fly development. *Wigglesworthia* and *Sodalis* densities were estimated using a quantitative PCR (qPCR) method performed on *Glossina palpalis gambiensis*, the vector of *Trypanosoma brucei gambiense* causing the chronic form of sleeping sickness, at different developmental stages.

2. Materials and methods

2.1. Tsetse flies

The *G. p. gambiensis* flies and pupae used in this experiment were randomly selected from a colony maintained in an insectary at CIRAD (Baillarguet, France), established from field fly populations collected in different areas in Burkina Faso 40 years ago. Following adult emergence, the population was maintained in a level 2 containment insectary at 23 °C and 80% relative humidity (Geiger et al., 2005) without any selection. Females were mated at day 3–4 postemergence with males aged 7–10 days postemergence.

2.2. DNA extraction from different stage tsetse fly samples

The total DNA of each sample was extracted using the protocol reported by Navajas et al. (1998). The teneral and old fly tissues (gut, salivary glands and ovaries) were dissected, pooled, and homogenized with a pestle in cetyl-trimethyl-ammonium bromide (CTAB) buffer (CTAB 2%; 0.1 M Tris, pH 8; 0.02 M EDTA, pH 8; 1.4 M NaCl). The pupae were homogenized entirely. The samples were incubated at 60 °C for 90 min. DNA was extracted from the lysis mixture with chloroform/isoamylic alcohol (24/1; V/V) and precipitated by adding isopropanol (V/V). After centrifugation (10,000*g*, 15 min), the pellet was rinsed with 70% ethanol, air-dried, and re-suspended in distilled sterile water and stored at −20 °C until use. Total DNA was extracted from flies at seven time points of their development: (a) at three different times for the pupae stage (at 24 and 48 h, corresponding to early deposition, and 28 days, late deposition), and (b) at the four following sampling times for adult flies: teneral, 2-week-old virgin adults, 4-week-old virgin adults, and 8-week-old virgin adults (48 h following their last blood meal); moreover, 2- and 8-week-old pregnant females were harvested (intrauterine larvae were removed from pregnant females' abdomen before DNA extraction). For each sampling time, the samples comprised, respectively, 15 pupae or 15 males plus 15 females (teneral and adult fly stage); DNA was extracted from each individual pupa or fly.

2.3. Design of primers for Sodalis glossinidius, Wigglesworthia glossinidia and Glossina palpalis gambiensis

Pairs of specific primers (Table 1) were designed using the sequences from the *Glossina morsitans morsitans tubulin* beta-1 gene (GenBank accession number, DQ377071), from the *Sodalis exochitinase* gene (GenBank accession number, Y11391), and from the *Wigglesworthia flagellin* (*fliC*) gene (GenBank accession number, DQ124689). These genes were chosen because they are single-copy genes. The different primers were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky, 2000).

The specificity of the primers was confirmed by sequencing. *Glossina* Tubulin F/R, *Sodalis* exochitinase F/R, and *Wigglesworthia* fliC F/R only amplified a region of the *G. p. gambiensis tubulin* gene, the *Sodalis exochitinase* gene, and the *Wigglesworthia fliC* gene, respectively; *Wigglesworthia* fliC primers did not amplify *Sodalis* DNA. All these primers were synthesized by Eurogentec (Eurogentec, France).

The different PCR products corresponding to the *Sodalis exochitinase*, *Wigglesworthia fliC*, and *G. p. gambiensis tubulin* genes were cloned into the pGEM-T Easy vector (Promega, Charbonnieres, France). For each gene, several recombinant plasmids were sequenced and compared with the reference sequences (GenBank accession numbers, DQ377071, Y11391, DQ124689).

2.4. Quantitative PCR assays and standard curves

Quantitative PCR amplifications were performed with a qPCR Stratagene MxPro 3005P (Stratagene, Paris, France) detection system using a standard protocol (95 °C, 10 min and 40 cycles: 95 °C, 30 s; 60 °C, 45 s; 72 °C, 1 min). Non-template controls (water) were included in each assay. The Brilliant II SYBR Green qPCR Master Mix (Stratagene, Paris, France) was used in a final volume of 25 µl. For each analysis, 2 µl of tenfold-diluted DNA was used as the initial template. After several trials, the primer concentrations were determined (Table 1). The specificity of the amplicons was assessed by checking the dissociation curve.

PCR amplicons, obtained by amplification of the *tubulin*, *exochitinase*, and *fliC* genes, respectively, using the primers described above (Table 1), were purified with the "Qiaquick purification kit" (Qiagen, Paris, France). The purified PCR products of each gene were used to set up standard curves. They were generated using tenfold serial dilutions of each purified PCR product of a known concentration. The slope and efficiency values of the standard