Bacterial flora as indicated by PCR-temperature gradient gel electrophoresis (TGGE) of 16S rDNA gene fragments from isolated guts of phlebotomine sand flies (Diptera: Psychodidae)

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INTRODUCTION

Insects harbor a rich and complex community of microorganisms in their guts and other body regions. This microbiota participates in many types of interactions ranging from pathogenesis to obligate mutualism (Dillon and Dillen 2004). Approximately 700 species of sand flies have been described to date, 10% of which have been incriminated as Leishmania vectors (Killick-Kendrick 1990). Female sand flies acquire the Leishmania parasite by biting an infected host. In the vector midgut, parasites multiply and transform from amastigotes to promastigotes. The infection of the mammalian host is initiated by multiply and transform from amastigotes to promastigotes by biting an infected host. In the vector midgut, parasites (2004) demonstrated that bacteria present in the digestive tract of Phlebotomus duboscqi induced the secretion of antimicrobial peptides with a significant antiparasitic activity. Some reports deal with the occurrence of bacteria in the guts of sand flies (Adler and Theodor 1929, Schlein et al. 1985, Schlein and Jacobson 1994, Sang and Chance 1993, Oliveira et al. 2000). Dillon et al. (1996) and Volf et al. (2002) demonstrated that the maximum prevalence of bacteria in Phlebotomus papatasii and Phlebotomus duboscqi is recorded two days after blood feeding. High diversity in the bacterial gut microbiota, associated with different populations of Phlebotomus argentipes and Lutzomyia longipalpis, was registered using culture-dependent methods (Hillesland et al. 2008, Gouveia et al. 2008).

Beyond these studies, not much is known about the origin of the adult gut bacterial flora, quantitative and qualitative changes in this flora during insect development, or the impact of midgut bacteria on Leishmania development. Although an in vitro dual culture method with Leishmania donovoni has suggested that some bacterial strains, isolated from female P. argenteus, are able to interfere with L. donovoni promastigotes (Muniaraj et al. 2008). Abundance and diversity of gut-associated bacteria can be affected by various factors, including feeding behavior. Male and female sand flies feed on natural sugars, such as nectar, sap, and aphid and coccid secretions (Young et al. 1980, Killick-Kendrick and Killick-Kendrick 1987). These sugars are the main sources of carbohydrates for adults. Furthermore, females also feed on blood. Sand fly larvae are terrestrial and feed on soil organic matter (Feliciangeli 2004). It is thus supposed that the diversity in their feeding behavior, as well...
as conditions that pre-imaginal stages encounter during their development, affect gut microbiota and could impact their overall capacity to sustain Leishmania development. Until now, their gut bacterial flora has been investigated on isolated or pooled guts via culture of bacterial gut content and the use of bacteriology or cloning and sequencing of 16S rDNA to identify them (Rajendran and Modi 1982, Hillesland et al. 2008, Gouveia et al. 2008). However, labor and expense make such methodologies unsuitable for larger scale studies attempting to simultaneously ascertain and compare the diversity and abundance of bacteria in a large number of individually isolated guts. Therefore, fingerprinting methods may represent an alternative that has not been explored in sand flies. Denaturing gel electrophoresis (DGE), including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and temporal temperature gel electrophoresis (TTGE) analysis of PCR-amplified 16S rDNA fragments have been employed as a powerful tool for genetic fingerprinting of microbial communities (Muyzer 1999, Muyzer and Smala 1998). The most obvious advantage of these technologies is the convenience of simultaneously analyzing communities of a large number of samples originating from different environments or monitoring the behavior of one community over time or under different disturbances (Muyzer and Smala 1998).

We thus tested the capacity of a TGGE-based fingerprinting of 16S rDNA PCR fragments to assess changes in bacterial composition in individual sand fly guts. We followed the bacterial content of guts from different life stages of a laboratory-reared colony of Phlebotomus dubosci and from a wild caught Phlebotomus papatasi population.

MATERIALS AND METHODS

We used 36 specimens belonging to two species of sand flies, Phlebotomus papatasi and Phlebotomus dubosci. P. papatasi (eight males and eight females) were captured using CDC light traps in September 2008 in Marrakech City, Morocco (31° 37’ 48N, 8° 0’ 0W, 450 m). P. dubosci (four males, four females, four 2nd instar larvae, four 4th instar larvae, and four pupae) came from a colony reared under standard conditions at 26° C (Prof. B. Pesson, Laboratory of Parasitology, Strasbourg, France). Larvae of P. dubosci were fed on a sterilized mixture of rabbit dung and rodent food. For male and female adults of P. dubosci, natural sources of sugar were replaced by a solution of 30% saccharose. Females of P. papatasi and P. dubosci used here were not blood fed. Sand flies were kept in 96% ethanol until their dissection.

Gut dissection

Isolation of sand fly guts was conducted in a sterile environment on a sterile glass slide. Before dissection, each fly was surface sterilized for 1 min in 70% ethanol, then rinsed in sterile phosphate buffered saline (PBS). The guts were carefully dissected in sterile PBS with a sterile needle and kept in an Eppendorf tube for subsequent DNA extraction. An Eppendorf tube containing sterile PBS open near the dissection area constituted our sterility control during the dissection process. This control was included for each specimen dissection. For species identification of P. papatasi, genitalia of males, and female spermathecae, were mounted in Canada balsam.

Temperature Gradient Gel Electrophoresis

DNA was extracted under sterile conditions, using a Qiaamp kit (Qiagen), according to the manufacturer’s protocol. Purity and quantity of DNA were ascertained with a NanoDrop instrument (ThermoScientific), and 40ng of DNA was used as a template for 16S rDNA amplification. Primers U968-GC (5’- CGC CCG GGG CCG GCC CCG GGC GGG GCG GCA CGG GGGG AAC GCG AAG AAC CTT AC -3’) and L1401 (5’-CGG TGT GTA CAA GAC CC -3’) were used to amplify the 433bp of the V6 to V8 regions of the bacterial 16S rDNA (Nübel et al. 1996). All the primers used in this study were HPLC purified. Amplifications were carried out using 50 μl reaction volume and the PCR mix containing 40 ng genomic DNA, 1X PCR buffer, 0.5 μM of each primer, 0.2 mM of each dNTP, 3% DMSO, and 0.02 U Phusion (Finzyme). Two controls were set up, one during extraction, and one negative PCR. Samples were amplified using the following program: 98° C for 45 s, and 35 cycles of 98° C for 10 s, 60° C for 30 s, 72° C for 30 s, and 72° C for 7 min last extension. Aliquots of 5 μl were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide in order to check the sizes, contamination, and the amount of the amplicons. For each sample, two PCR products were pooled and concentrated in a speed vac to achieve a final concentration of about 700 ng/μl. TGGE was performed on a TGE Maxi system (Biometra). Denaturing gels (6% polyacrylamide gel electrophoresis with 20% deionized formamide, 2% glycerol, and 8 M urea) were made and run with 1x Tris-acetate-EDTA buffer. The temperature gradient (32° C to 55° C) was optimized for efficient separation of bands. The gels were run at 110 v for 18 h. Gel bands were visualized with a silver staining kit (Fermentas). Individual bands on silver-stained TGGE gels were picked up with sterile pipette tips, placed in 10 μl of sterile water, and directly used for reamplification. PCR products were purified by gel running and extracted with the QIAEXII kit (Qiagen) and were sequenced. The resulting sequences were compared with the non-redundant GenBank database using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

Figure 1 shows a representative gel of the bacterial TGGE profiles obtained during the study. The bacterial 16S rDNA gene was amplified from dissected guts of all sand fly stages and species studied, demonstrating therefore that all specimens, including males, bear bacteria in their gut. We observed that bacterial TGGE profiles, produced from isolated guts are reproducible between replicate samples (Figure 1A).
from a single gut dissected from 2nd and 4th instar larvae, samples (two bands for as described in the text. Figure 1A. The V6 to V8 region of the 16S rDNA was amplified stages (five bands) (Figure 1A). Of a total of seven separate bands identifiable from all studied guts, only one (band two) is present in all samples (Figure 1A). These results not only pinpoint the existence of major differences in the bacterial gut content between larval and adult sand fly life stages but also between adult P. duboscqi and P. papatasi. These differences are readily monitored by TGGE fingerprinting. We observed no differences in TGGE profiles between males and females of both Phlebotomus species. To our knowledge, this is the first report about bacterial flora from male sand flies. Bacterial identification performed after sequencing of the individual TGGE bands and a search for similarity in the existing GenBank database entries indicates that bacteria belonging to the genera Microbacterium and Chloroflexi, and to an unidentified bacterium, are present in the gut of sand flies (Figure 1B). The band referenced as two in Figure 1A, common to all the TGGE profiles generated, was identified as Microbacterium spp. The presence of Microbacterium species was also reported in the guts of field trapped P. argentipes from India and of Ixodes ricinus (Hillesland et al. 2008, Rudolf et al. 2009). The presence of Microbacterium spp. in all analyzed samples raises questions about the nature of the interaction between this microorganism and sand flies. Interestingly, various strains of Microbacterium isolated from Musca domestica guts have been shown to support its larval development (Zurek et al. 2000). To what extent it could play a similar role during sand fly larval development remains unknown and has to be investigated. In previous studies using a culture dependent methodology, Volf et al. (2002) detected Ochrobactrum sp. as the dominant bacteria within larvae, pupae and newly-emerged females of a laboratory-reared colony of P. duboscqi. They then suggested a transstadial passage of this bacterium from larvae to adults. Within Anopheles species, 90% of newly-emerged An. gambiae and 73% of newly-emerged An. stephensi were found to host bacteria and transstadial transmission was experimentally demonstrated by a successful passage of an Escherichia coli strain from larvae to adults (Pumpuni et al. 1996). In this study, the presence of Microbacterium in the 2nd and 4th instar larval, pupae, and male and female adults of P. duboscqi could be the result of a transstadial transmission. This is the first time that bacteria belonging to the Chloroflexi genera are identified in sand fly guts, although only in the pre-imaginal stages. These bacteria have been previously characterized in communities sampled from various soil environments and more recently in the gut of the soil-feeding termite, Cubitermes niokoloensis (Zhang et al. 2007, Fall et al. 2007). Our data suggest that bacteria associated with the sand fly gut belong to a group that was previously characterized in a soil bacterial consortium (Zhang et al. 2007, Park et al. 2008).

In conclusion, TGGE analysis of PCR-amplified 16S rDNA fragments allowed us to demonstrate and follow the major reorganization of the gut bacterial community that occurs during metamorphosis of sand flies. This methodology, applied on isolated single sand fly guts, highlighted differences and similarities between species, sexes, and life stages of sand flies. Its ability to integrate uncultured bacteria is a further asset of this method. The use of such methodology in conjunction with other culture-based methodologies will be of great help in investigating the behavior of the Leishmania-bacterial community in its ecological context.

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REFERENCES CITED


