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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)

S. Guernaoui¹✉, D. Garcia¹, E. Gazanion¹, Y. Ouhdouch², A. Boumezzough³, B. Pesson⁴, D. Fontenille¹, and D. Sereno¹✉

¹UR16 Institut de Recherche pour le Développement BP 64501, 34394 Montpellier Cedex 5, Montpellier, France

²Laboratoire de Biologie et Biotechnologie des Microorganismes, Faculté des Sciences Semlalia, Université Cadi Ayyad, Département de Biologie, Avenue Prince My. Abdellah, BP 2390, 40000 Marrakech, Morocco

³Laboratoire Ecologie- Environnement, Faculté des Sciences Semlalia, Université Cadi Ayyad, Département de Biologie, Avenue Prince My. Abdellah, BP 2390, 40000 Marrakech, Morocco

⁴Laboratoire Parasitologie, Université Strasbourg I, Faculté de Pharmacie, 74 route du Rhin, BP 24, 67401 Illkirch, France

ABSTRACT: In this study, we tested the capacity of Temperature Gradient Gel Electrophoresis (TGGE)-based fingerprinting of 16S rDNA PCR fragments to assess bacterial composition in a single isolated sand fly gut. Bacterial content was studied in different life stages of a laboratory-reared colony of *Phlebotomus duboscqi* and in a wild-caught *Phlebotomus papatasi* population. Our study demonstrates that a major reorganization in the gut bacterial community occurs during metamorphosis of sand flies. *Chloroflexi* spp. was dominant in the guts of pre-imaginal stages, although *Microbacterium* spp. and another as yet unidentified bacteria were detected in the gut of the adult specimen. Interestingly, *Microbacterium* spp. was also found in all the adult guts of both species. We demonstrate that the analysis of bacterial diversity in an individualized sand fly gut is possible with fingerprinting of 16S rDNA. The use of such methodology, in conjunction with other culture-based methods, will be of great help in investigating the behavior of the *Leishmania*-bacterial community in an ecological context. **Journal of Vector Ecology 36 (Supplement 1): S144-S147. 2011.**

Keyword Index: Phlebotomine sand flies, gut, bacteria, temperature gradient gel electrophoresis (TGGE).

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 Dillon 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 metacyclics, which are introduced into the skin during a subsequent blood meal.

The sand fly gut, where *Leishmania* permanently resides, represents an important interface for vector-parasite interaction. In this compartment, *Leishmania* have to co-exist with other microorganisms, including bacteria. Indeed, in mosquitoes, midgut bacterial infections significantly affect sporogonic development of *Plasmodium* via the activation of the immune response (Pumpuni et al. 1996, Meister et al. 2009, Dong et al. 2009). Boulanger et al. (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 papatasi* 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 donovani* has suggested that some bacterial strains, isolated from female *P. argentipes*, are able to interfere with *L. donovani* 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 (Felicangeli 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 duboscqi* 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 duboscqi*. *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. duboscqi* (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. duboscqi* were fed on a sterilized mixture of rabbit dung and rodent food. For male and female adults of *P. duboscqi*, natural sources of sugar were replaced by a solution of 30% saccharose. Females of *P. papatasi* and *P. duboscqi* 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 Qiap 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 CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG 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 (Fininzyne). 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 TGGE 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).

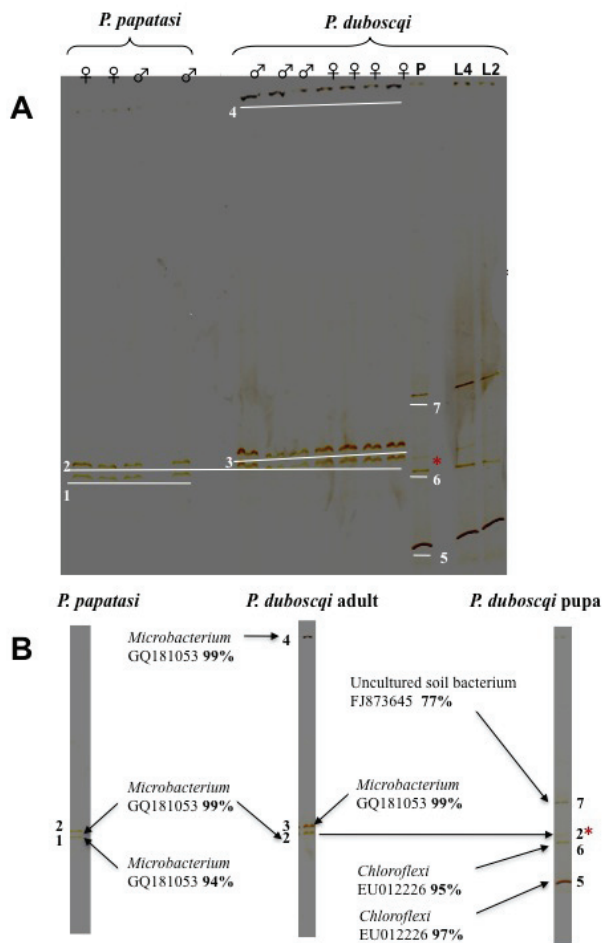


Figure 1. The V6 to V8 region of the 16S rDNA was amplified as described in the text. (A) Representative TGGE profiles from a single gut dissected from 2nd and 4th instar larvae, pupae, and male and female adults of *P. duboscqi* and male and female adult *P. papatasi*. (B) Sequence assignment of bacteria.

The number of distinguishable bands in the adult gut samples (two bands for *P. papatasi* and three bands for *P. duboscqi*) was significantly lower than from pre-imaginal 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 transtadial 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 transtadial 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 larvae, pupae, and male and female adults of *P. duboscqi* could be the result of a transtadial 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

Adler, S. and O. Theodor. 1929. Attempts to transmit *Leishmania tropica* by bite: the transmission of *L. tropica* by *Phlebotomus sergenti*. Ann. Trop. Med.

- Parasitol. 23: 1-18.
- Boulanger, N., C. Lowenberger, P. Volf, R. Ursic, L. Sigutova, L. Sabatier, M. Svobodova, S.M. Beverley, G. Spath, R. Brun, B. Pesson, and P. Bulet. 2004. Characterization of a defensin from the sand fly *Phlebotomus duboscqi* induced by challenge with bacteria or the protozoan parasite *Leishmania major*. *Infect. Immun.* 72: 7140-7146.
- Dillon, R.J., E. El Kordy, M. Shehata, and R.P. Lane. 1996. The prevalence of a microbiota in the digestive tract of *Phlebotomus papatasi*. *Ann. Trop. Med. Parasitol.* 90: 669-673.
- Dillon, R.J. and V.M. Dillon. 2004. The gut bacteria of insects: nonpathogenic interactions. *Annu. Rev. Entomol.* 49: 71-92.
- Dong, Y., F. Manfredini, and G. Dimopoulos. 2009. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathol.* 5: e1000423.
- Fall, S., J. Hamelin, F. Ndiaye, K. Assigbetse, M. Aragno, J.L. Chotte, and A. Brauman. 2007. Differences between bacterial communities in the gut of a soil-feeding termite (*Cubitermes niokoloensis*) and its mounds. *Appl. Environ. Microbiol.* 16: 5199-5208.
- Feliciangeli, M.D. 2004. Natural breeding places of phlebotomine sand flies. *Med. Vet. Entomol.* 18: 71-80.
- Gouveia, C., M.D. Asensi, V. Zahner, E.F. Rangel, and S.M. Oliveira. 2008. Study on the bacterial midgut microbiota associated to different Brazilian populations of *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae). *Neotrop. Entomol.* 37: 597-601.
- Hillesland, H., B. Read, B. Subhadra, I. Hurwitz, R. McKelvey, K. Ghosh, P. Das, and R. Durvasula. 2008. Identification of aerobic gut bacteria from the kala azar vector, *Phlebotomus argentipes*: a platform for potential paratransgenic manipulation of sand flies. *Am. J. Trop. Med. Hyg.* 79: 881-886.
- Killick-Kendrick, R. and M. Killick-Kendrick. 1987. Honeydew of aphids as a source of sugar for *Phlebotomus ariasi*. *Med. Vet. Entomol.* 1: 297-302.
- Meister, S., B. Agianian, F. Turlure, A. Relgio, I. Morlais, F.C. Kafatos, and G.K. Christophides. 2009. *Anopheles gambiae* PGRPLC-mediated defense against bacteria modulates infections with malaria parasites. *PLoS Pathol.* 5: e1000542.
- Muniaraj, M., D.S. Dinesh, P.K. Sinha, P. Das, and S.K. Bhattacharya. 2008. Dual culture method to determine the relationship of gut bacteria of sand fly (*Phlebotomus argentipes*) with promastigotes of *Leishmania donovani*. *J. Commun. Dis.* 40: 133-138.
- Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr. Opin. Microbiol.* 2: 317-322.
- Muyzer, G. and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek.* 73: 127-141.
- Nübel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R.I. Amann, W. Ludwig, and H. Backhaus. 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.* 178: 5636-5643.
- Oliveira, S.M., B.A. Moraes, C.A. Gonçalves, C.M. Giordano-Dias, J.M. D'Almeida, M.D. Asensi, R.P. Mello, and R.P. Brazil. 2000. Prevalence of microbiota in the digestive tract of wild females of *Lutzomyia longipalpis* Lutz & Neiva, 1912 (Diptera: Psychodidae). *Rev. Soc. Bras. Med. Trop.* 33: 319-322.
- Park, M.J., M.K. Kim, H.B. Kim, W. Im, T.H. Yi, S.Y. Kim, N.K. Soung, and D.C. Yang. 2008. *Microbacterium ginsengisoli* sp. nov., a β -glucosidase-producing bacterium isolated from soil of a ginseng field. *Int. J. Syst. Evol. Microbiol.* 58: 429-433.
- Pumpuni, C.B., J. Demaio, M. Kent, J.R. Davis, and J.C. Beier. 1996. Bacterial population dynamics in three anopheline species: the impact on *Plasmodium* sporogonic development. *Am. J. Trop. Med. Hyg.* 54: 214-218.
- Rajendran, P. and G.B. Modi. 1982. Bacterial flora of sand fly gut (Diptera: Psychodidae). *Ind. J. Publ. Hlth.* 26: 49-52.
- Rudolf, I., J. Mendel, S. Sikutová, P. Svec, J. Masaríková, D. Nováková, L. Bunková, I. Sedláček, and Z. Hubálek. 2009. 16S rRNA gene-based identification of cultured bacterial flora from host-seeking *Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis concinna* ticks, vectors of vertebrate pathogens. *Folia Microbiol. (Praha)* 54: 419-428.
- Sang, D.K. and M.L. Chance. 1993. Studies on the *Phlebotomus* fauna of Mount Elgon, Kenya. *Ann. Trop. Med. Parasitol.* 87: 509-515.
- Schlein Y. and R.L. Jacobson. 1994. Mortality of *Leishmania major* in *Phlebotomus papatasi* caused by plant feeding of the sand flies. *Am. J. Trop. Med. Hyg.* 50: 20-27.
- Schlein, Y., I. Polacheck, and B. Yuval. 1985. Mycoses, bacterial infections and antibacterial activity in sand flies (Psychodidae) and their possible role in the transmission of leishmaniasis. *Parasitology* 90: 57-66.
- Volf, P., A. Kiewegovo, and A. Nemeč. 2002. Bacterial colonisation in the gut of *Phlebotomus duboscqi* (Diptera: Psychodidae): transtadial passage and the role of female diet. *Folia Parasitol. (Praha)* 49: 73-77.
- Young, C.J., D.P. Turner, R. Killick-Kendrick, J.A. Rioux, and A.J. Leaney. 1980. Fructose in wild-caught *Phlebotomus ariasi* and the possible relevance of sugars taken by sand flies to the transmission of leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.* 74: 363-366.
- Zhang, H.B., W. Shi, M.X. Yang, T. Sha, and Z.W. Zhao. 2007. Bacterial diversity at different depths in lead-Zinc mine tailings as revealed by 16S rRNA gene libraries. *J. Microbiol.* 45: 479-484.
- Zurek, L., C. Schal, and D.W. Watson. 2000. Diversity and contribution of the intestinal bacterial community to the development of *Musca domestica* (Diptera: Muscidae) larvae. *J. Med. Entomol.* 37: 924-928.