Chikungunya virus impacts the diversity of symbiotic bacteria in mosquito vector

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Abstract

Mosquitoes transmit numerous arboviruses including dengue and chikungunya virus (CHIKV). In recent years, mosquito species Aedes albopictus has expanded in the Indian Ocean region and was the principal vector of chikungunya outbreaks in La Reunion and neighbouring islands in 2005 and 2006. Vector-associated bacteria have recently been found to interact with transmitted pathogens. For instance, Wolbachia modulates the replication of viruses or parasites. However, there has been no systematic evaluation of the diversity of the entire bacterial populations within mosquito individuals particularly in relation to virus invasion. Here, we investigated the effect of CHIKV infection on the whole bacterial community of Ae. albopictus. Taxonomic microarrays and quantitative PCR showed that members of Alpha- and Gammaproteobacteria phyla, as well as Bacteroidetes, responded to CHIKV infection. The abundance of bacteria from the Enterobacteriaceae family increased with CHIKV infection, whereas the abundance of known insect endosymbionts like Wolbachia and Blattabacterium decreased. Our results clearly link the pathogen propagation with changes in the dynamics of the bacterial community, suggesting that cooperation or competition occurs within the host, which may in turn affect the mosquito traits like vector competence.

Keywords: Aedes albopictus, bacterial community, chikungunya, microarray, quantitative PCR, Wolbachia

Introduction

Mosquitoes are major vectors of arboviruses. In the last few decades, the mosquito species Aedes albopictus, originating from Southeast Asia (Smith, 1956), has expanded over the world and has been responsible for many epidemic outbreaks of chikungunya and dengue (reviewed in Paupy et al. 2009). For example, Ae. albopictus has been identified as the principal vector of chikungunya virus (CHIKV) in regions of the Indian Ocean (Delatte et al. 2008a,b; Bagny et al. 2009a,b), Asia (Manimunda et al. 2010), Africa (Peyrefitte et al. 2007, Peyrefitte et al. 2008) and Europe (Rezza et al. 2007). Massive chikungunya outbreaks have been correlated with an emerging CHIKV variant that is efficiently replicated and transmitted by Ae. albopictus (Schuffenecker et al. 2006; Vazeille et al. 2007; Dubrulke et al. 2009; Tsetsarkin et al. 2011).

Over the last few years, many studies have focused on the impact microbial communities have on the fitness and competence of the insect vectors of pathogens. It has been established that microbial symbionts can contribute to insect host adaptation, including the aspects of nutrition and survival (Moya et al. 2008; Oliver et al. 2010), reproduction (reviewed in Werren et al. 2008), heat tolerance (Montillor et al. 2002), protection against pathogens or natural enemies (Scarborough et al. 2005; Hedges et al. 2008; Oliver et al. 2009) and vector competence (Gottlieb et al. 2010). Particularly, the bacterium Wolbachia was shown to interfere with or modulate the replication and transmission of both arboviruses and parasites.
in mosquitoes (Kambris et al. 2009; Moreira et al. 2009; Bian et al. 2010; Glaser & Meola 2010; Mousson et al. 2010; Walker et al. 2011). Microbiota endogenous to some mosquito species induce the expression of basal genes in the antimicrobial immune response, thus optimizing host immune signalling when pathogens attack. For instance, the microbiota of mosquito vector *Aedes aegypti* influence the infection by the dengue virus by modulating the gene expression of several antimicrobial peptides (Xi et al. 2008). In addition, modification or elimination of commensal microbiota increased the sensitivity of *Anopheles gambiae* to *Plasmodium falciparum* (Dong et al. 2009). Altogether, these data indicate that there is a key role of resident bacterial communities and invading pathogens in the mosquito vector pathosystem.

To better understand the mechanisms involved in the failure or success of pathogen propagation and maintenance in the insect host, the interaction between resident microbes and invading pathogen needs to be taken into account not only at the individual or the species level, but also in terms of the ecological community unit. Advances in this domain are impeded by a weak knowledge of the microbial diversity occurring in insect host. Some studies have begun describing the bacterial community associated with mosquito vectors. For *Aedes* mosquitoes, cultivable bacteria belonging to *Enterobacteriaceae* family as *Enterobacter*, *Klebsiella* and *Serratia* have been reported in the midgut of *Aedes triseriatus* maintained in the laboratory (Demaio et al. 1996). Recently, wild populations of *A. albopictus* and *A. aegypti* have been shown to harbour principally *Proteobacteria* and *Firmicutes*, including genera *Acinetobacter*, *Asaia*, *Delftia*, *Pseudomonas*, *Wolbachia* and *Bacillus*, as well as members of the family *Enterobacteriaceae* (Zouache et al. 2011). Although mosquito-associated bacteria have recently been shown to affect the transmission of pathogens, few studies have tried to link the pathogen infections with alterations in the insect-vector microbiota composition. Here, the effect of CHIKV infection on the bacterial community in the *Ae. albopictus* population from La Rénunion was investigated at different times after a bloodmeal. The dynamics of change in bacterial community was investigated at different times after a bloodmeal. Results showed that CHIKV impacts the diversity of symbiotic bacteria in the mosquito vector.

**Material and methods**

**Mosquito rearing**

Larvae of *Aedes albopictus* were collected in Providence, La Réunion Island, and maintained in insectaries. This strain ALPROV is naturally infected with *Wolbachia* strains *wAlbA* and *wAlbB* (Tortosa et al. 2008; Zouache et al. 2009) and other commensal bacteria (Zouache et al. 2009). The *Ae. albopictus* ALPROV population was maintained in standard conditions as described (Mousson et al. 2010), and the fourth generation was used for all experiments. Briefly, eggs from the third generation were hatched in water, and larvae were reared in pans containing one yeast tablet per litre of dechlorinated tap water. Adult mosquitoes were fed with 10% sucrose and maintained at 28 ± 1 °C and 80% relative humidity under 16-h light/8-h dark cycles.

**Virus production and oral infection**

The strain CHIKV (E1-226V), which has an A-V amino acid substitution at position 226 in the E1 glycoprotein, was used (Schuffenecker et al. 2006). Virus stock and mosquito infection were performed as described (Vazeille et al. 2007; Mousson et al. 2010). To infect mosquitoes, 1 mL of viral suspension was added to 2 mL of washed rabbit erythrocytes supplemented with ATP (5 × 10⁻³ M) as a phagostimulant. The resulting infectious blood at a titre of 10⁷·⁵ PFU/mL was transferred to a glass feeder at 37 °C on top of the mesh of a plastic box containing 60 1-week-old female mosquitoes that had been starved for 24 h. After 15 min of feeding, mosquitoes were sorted on ice. Engorged females were transferred to cardboard containers, then fed with 10% sucrose at 28 °C and analysed daily for 14 days.

**Nucleic acid extraction**

Five female mosquitoes were killed each day until day 14 post-infection. RNA and DNA were co-extracted from individual mosquitoes using the NucleoSpin® RNA/DNA buffer set and NucleoSpin® RNA II, following the manufacturer’s instructions (Macherey-Nagel). Each mosquito was crushed in 350 µL of lysis buffer (RA1) with 1% β-mercaptoethanol. Samples were homogenized through a NucleoSpin filter. After adding 350 µL of 70% ethanol, samples were transferred to NucleoSpin columns. Nucleic acids were bound to the column membrane by centrifugation at 11 000 g for 30 s. After 2 washes, DNA was eluted with 60 µL of water. Column membranes were treated with DNase (according to the manufacturer’s instructions), and after successive washes, RNA was eluted with 40 µL of RNAse-free water. DNA and RNA were quantified using an ND-1000 spectrophotometer (NanoDrop Technologies) and stored at −20 °C (DNA) or −80 °C (RNA) until use.

**Quantifying chikungunya virus**

RNA extracted from one mosquito was used to measure the viral load by quantitative RT-PCR. One-step
RT–PCR was performed in the LightCycler apparatus (Roche Applied Science) using EXPRESS One-Step SYBR®GreenER™ Kit (Invitrogen) in a volume of 20 μL containing 10 ng of RNA template, 1x EXPRESS SYBR® GreenER™ SuperMix Universal, 200 nm of each primer and 1x EXPRESS Superscript® Mix. Primers used correspond to the E2 structural protein coding region: Chik/E2/9018/+ (5’-CACCCGCAGCAACTACCG-3’) and Chik/E2/9235/- (5’-GATTGTGACCCGCCGCA-3’). The amplification programme was 50 °C for 15 min, 95 °C for 2 min, then 40 cycles of 95 °C for 15 s and 63 °C for 1 min. Viral RNA was quantified by comparison to a standard curve generated using duplicates of a 10-fold serial dilution of synthetic CHIKV RNA transcripts (Mousson et al. 2010).

**PCR detection and quantification of Wolbachia and the total bacterial community**

Relative levels of the wsp gene, indicative of Wolbachia, the rrs gene, indicative of the total bacterial community, and the mosquito host actin gene in the DNA extracted from mosquitoes were measured by quantitative PCR. DNA was amplified using the LightCycler LC480 apparatus (Roche Applied Science). The 20-μL reaction mixture contained 5 ng of template DNA, 1x LightCycler DNA Master SYBR Green I (Roche Applied Science) and primers for wsp and rrs at 300 nm or for actin at 200 nm. Primers used were as follows: QAdir1 (5’-GGG TTGATGTTGAAGGAG-3’) and QArev2 (5’-CACCAGC TTTACTTGACC-3’) for the wAlbA gene; 183F (5’-AA GGAACCGAAGTTCATG-3’) and QBrev2 (5’-AGTTGT GAGTAATGTCCC-3’) for the wAlbB gene; actAlb-dir (5’-GCACACGATGATCCCTGAC-3’) and actAlb-rev (5’-GTCAGGAGAACTGCGTGCT-3’) for the actin gene; 519F (5’-CAGCMCGCCCCTGTAANW-3’) and 907R (5’- CCGTCAATTCMTTRAGTT-3’) for the rrs gene (numbers correspond to equivalent positions in Escherichia coli gene) (Lane 1991). The PCR programme was 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 60 °C (for wsp and actin genes) or 63 °C (for the rrs gene) and 72 °C for 30 s. Standard curves were drawn of the amplification from template pQuantAlb16S, the pQuantAlb plasmid (Tortosa et al. 2008) containing 5 ng of template DNA, 1x ExpressOne-Step RT–PCR was performed in a T gradient Thermocycler (Biometra) using a 50-μL reaction mixture containing 1 μL of DNA template in 1x polymerase reaction buffer (Roche Applied Science), 200 μM of each deoxynucleoside triphosphate, 500 nm of each primer, 0.025 mg/mL of T4 gene protein 32 (Roche Applied Science) and 0.5 U of Expand polymerase (Roche Applied Science). PCR products were purified using the MinElute PCR purification kit (Qiagen, Hilden, Germany). DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and adjusted to 50 ng/μL. PCR products containing the T7 promoter were used as templates for T7 RNA polymerase–mediated in vitro transcription (Stralis-Pavese et al. 2004) and labelled with Cy3. Labelling was carried out as previously described (Kyselková et al. 2008). Briefly, RNA (50 μL) was incubated at 60 °C for 30 min with 5.71 μL of 100 mm ZnSO4 and 1.43 μL of 1 mm Tris-Cl (pH 7.4). Fragmentation was stopped on ice by adding 1.43 μL of 500 mm EDTA (pH 8). RNAsin (40 U) was added. Fragmented and labelled RNA was stored at −20 °C until use.

**Probe and microarray manufacture**

Probes targeting different taxonomic levels such as phylum, family, genus and species were designed using ARB software and its rrs database (Ludwig et al. 2004; SSURel_96_SILVA_04_10_08_opt.arb; http://www.arb-home.de/). Probes used were those designed by Sangüin et al. (2006a,b, 2008) and Kyselková et al. (2009). Probes synthesized in vitro (Eurogentec, Seraing, Belgium) were spotted onto slides at 50–55% relative humidity and 19 °C with a MicroGrid II spotter (BioRobotics, Cambridge, UK). Each probe was spotted four times per slide. Slides were incubated overnight at room temperature in the spotter, placed for 15 min in a humid Genetix hybridisation chamber (Proteogene, St Marcel, France) at 37 °C, dried at 120 °C for 90 min, then stored in the dark at room temperature.

**Hybridization**

Slides were washed twice for 2 min each in 0.2x sodium dodecyl sulphate (SDS) and twice for 2 min each in filtered deionized water with vigorous shaking at room temperature. Slides were incubated for 15 min in a fresh blocking solution (0.25 g of NaBH4 in 75 mL of PBS and 25 mL of absolute ethanol) to reduce all remaining reactive aldehyde groups on the slides. Hybridization was carried out as described previously (Kyselková et al. 2008).
Scanning, image analysis and normalization

Slides were scanned at 532 nm with a 10-μm resolution using a GeneTac LS IV scanner (Genomic Solutions, Huntingdon, UK). Images were analysed using GENEPIX 4.01 software (Axon, Union City, CA, USA). Data were filtered using R 2.10.0 software (R Development Core Team, 2009; http://www.r-project.org) with parameters defined by Sanguin et al. (2006a,b).

Statistical analysis

All statistical analyses were carried out using R software version 2.10.0 (R Development Core Team, 2009; http://www.r-project.org). Differences in CHIKV titre and densities of Wolbachia and total bacteria in Aedes albopictus were tested using ANOVA and Tukey HSD (honestly significant difference) according to the infection status, day and their interaction as fixed effects. The proportion of variance in hybridization data because of the infection status and time was tested in a 50–50 multivariate analysis of variance (FF-MANOVA) with 10 000 rotations with infection and time as fixed effects (Langsrud 2002, 2005).

To determine the correlation between a given probe and infection status, conditional inference random forest (CI-RF) prediction was performed using the RAND-FORESTGUI program version 1.1 developed for the R environment (R. J. Michelland & G. L. Grundmann, unpublished data). A CI-RF is an ensemble of individual conditional inference random forest (CI-RF) prediction was performed using the RAND-FORESTGUI program version 1.1 developed for the R environment (R. J. Michelland & G. L. Grundmann, unpublished data). A CI-RF is an ensemble of individual conditional inference random forest as base learners, considered to be unbiased in comparison with classification and regression analysis trees (CART) (Strobl et al. 2007). For each sample, each individual tree votes for one cluster and the forest predicts the cluster that has the most votes. The rate of sample misclassification in good clusters by the forest is termed the out-of-bag error (OOB). The CI-RF was fitted using (i) the microarray data of the 3 dates post-infection (days 5, 9 and 14 post-infection) corresponding to the maximum virus load; (ii) the trees were not pruned and were grown as large as possible; (iii) the number of randomly selected probes to be searched through to find the best split at each node (mtry) was the square root of the number of probes; and (iv) the importance of probes used was the unbiased conditional mean decrease in accuracy proposed by Strobl et al. (2007). The dissimilarity between samples was plotted using an isotonic non-metric multidimensional scaling (nMDS) with 10 000 random starts.

To identify the smallest set of non-redundant probes involved in the discrimination of infection status, we carried out a backward probe elimination algorithm from supervised CI-RF by successively eliminating the least important probes and by using OOB as the minimization criterion. To comfort the robustness of this probe set, two subsequent analyses were performed: (i) the Pearson $r^2$ value was calculated between the initial CI-RF and the new CI-RF dissimilarities and (ii) an iterative Kruskal–Wallis test was performed for each probe. The selected probes were plotted using a heatmap based on Ward trees with the CI-RF dissimilarities to cluster microarray samples and Euclidian distances to cluster probes. To test the changes in microarray probe intensity over time, the Kruskal–Wallis test was performed for infected and uninfected mosquito data.

Results

CHIKV replication

After an infectious bloodmeal, the CHIKV viral load in the ALPROV line of Aedes albopictus increased significantly from $10^{4.54} ± 10^{0.06}$ at day 0 to a maximum of $10^{8.36} ± 10^{0.24}$ at day 5 post-infection ($P < 0.001$) (Fig. 1). The viral load remained high until day 8 post-infection ($10^{8.10} ± 10^{0.05}$), decreasing slightly until day 14 post-infection ($10^{7.50} ± 10^{0.18}$).

Total bacterial community

Bacterial density, as measured by the ratio of bacterial rrs to host actin gene copies (Fig. 2), was not significantly different between CHIKV-infected and uninfected mosquitoes. The copy number of the bacterial rrs gene per mosquito at day 0 was between $10^{1.34} ± 10^{0.07}$ (infected) and $10^{1.36} ± 10^{0.09}$ (uninfected) and at day 13 between $10^{1.54} ± 10^{0.14}$ (uninfected) and $10^{1.58} ± 10^{0.16}$ (infected) ($P > 0.01$). Although not significant, the

Fig. 1 Variation in chikungunya virus load. Five Aedes albopictus ALPROV females were killed each day post-infection and nucleic acids extracted. RNA was used to measure the mean viral load by quantitative RT–PCR. Bars represent ± standard deviation (SD).
bacterial density tended to increase slightly from day 11 to day 13 in both CHIKV-infected and uninfected mosquitoes.

A taxonomic microarray was used to analyse the effects of blood feeding and CHIKV infection on bacterial community in mosquitoes by comparing rrs gene sequences present in mosquitoes 1 day before and at days 0, 2, 5, 9 and 14 after an infected or uninfected bloodmeal. In total, 131 of 1035 probes were positive (12.8%). Of these, most probes (73.3%) corresponded to sequences from bacteria belonging to Proteobacteria comprising Alpha (38.5%), Beta (21.9%), Gamma (31.3%), Delta (6.2%) and Epsilon (2.1%) classes. Other positive probes corresponded to sequences from phyla Firmicutes (11.5%), Bacteroidetes (4.6%), Actinobacteria (2.25%), Acidobacteria (3.0%), Cyanobacteria (2.3%), Planctomycetes (0.8%) and OP11 division (2.25%).

The average number of positive probes per mosquito was 42.7 ± 7.1. FF-MANOVA showed that kinetics and infection status explained 38% and 5% of the variance, respectively (P < 0.001).

To compare the infected and uninfected modalities, mosquitoes on days 5, 9 and 14 after the bloodmeal were chosen as times corresponding to the maximum virus load (Fig. 1). A supervised CI-RF of the microarray data set was performed with the 131 positive probes using time, infection status and their interaction as fixed effects. To ensure the best performance of the supervised CI-RF, the best entry for the model was previously determined as 13 (see Material and methods). Results showed a good discrimination between infected and uninfected samples (Fig. 3). The OOB of 4% of the supervised CI-RF was low because of one sample misclassified in the uninfected clusters (D09_IN) (Fig. 3).

To identify the set of non-redundant probes significantly involved in the discrimination of samples, a backward probe elimination algorithm was applied. This procedure allowed successive elimination of the least important probes using OOB as minimization criterion (Fig. S1, Supporting Information), and identified 15 discriminating probes with the smallest OOB of 4% (Table 1, Fig. S1A, Supporting Information). This OOB value was explained by the same misclassification of D09_IN as in the initial supervised CI-RF. The correlation between CI-RF dissimilarities using 131 probes or the 15 discriminating probes was 0.96 (P < 0.001, Fig. S1B, Supporting Information), which demonstrated that these 15 probes were sufficient to classify the samples. The Kruskal–Wallis test performed on each of the 15 probes showed a significant difference between infected and uninfected clusters (P < 0.05).

Variation in bacterial groups after chikungunya virus infection

The contribution of each of the 15 probes in discriminating uninfected from CHIKV-infected mosquitoes ALPROV was assessed (Fig. 4). The relative intensities of Gammaproteobacteria probes were higher for CHIKV-infected individuals (P < 0.001). The signal intensity of probes targeting sequences from bacteria affiliated to...
Table 1 Phylogenetic affiliation of the target of the 15 discriminating probes

<table>
<thead>
<tr>
<th>Probes</th>
<th>Class</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
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<tbody>
<tr>
<td>Alpha685</td>
<td>Alphaproteobacteria</td>
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<tr>
<td>Brady4</td>
<td>Alphaproteobacteria</td>
<td>Bradyrhizobiaceae</td>
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<tr>
<td>Wol3</td>
<td>Alphaproteobacteria</td>
<td>Anaplasmataceae</td>
<td>Wolbachia</td>
<td>pipientis</td>
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<tr>
<td>Wol4</td>
<td>Alphaproteobacteria</td>
<td>Anaplasmataceae</td>
<td>Wolbachia</td>
<td>pipientis</td>
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<tr>
<td>Rhodobacter1B</td>
<td>Alphaproteobacteria</td>
<td>Rhodobacteriaceae</td>
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<tr>
<td>Blatta2</td>
<td>Bacteroidetes</td>
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<td>Ecoli1B</td>
<td>Gammaproteobacteria</td>
<td>Enterobacteriaceae</td>
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<tr>
<td>enter3</td>
<td>Gammaproteobacteria</td>
<td>Enterobacteriaceae</td>
<td>Escherichia</td>
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<td>Gammaproteobacteria</td>
<td>Enterobacteriaceae</td>
<td>Escherichia</td>
<td>coli, alberti</td>
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<tr>
<td>enter5</td>
<td>Gammaproteobacteria</td>
<td>Enterobacteriaceae</td>
<td>Escherichia</td>
<td>Citrobacter, Salmonella, Shigella</td>
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<tr>
<td>enter6</td>
<td>Gammaproteobacteria</td>
<td>Enterobacteriaceae</td>
<td>Escherichia</td>
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<td>Enterobacteriaceae</td>
<td>Pantoea</td>
<td>agglomerans</td>
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<td>Plancto12</td>
<td>Planctomycetes</td>
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<tr>
<td>OP2-1 (OP2 phylum)</td>
<td>Uncultured Eubacteria</td>
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<tr>
<td>OP11-5 (OP11 division)</td>
<td>Uncultured Eubacteria</td>
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</table>

Fig. 4 Heatmap of the 15 positive bacterial rRNA probes discriminating CHIKV-infected from uninfected AL-PROV mosquitoes. Samples are clustered using the Ward tree method based on supervised CI-RF dissimilarities, while probes are clustered using the Euclidian distances. Relative fluorescent intensity of probes is indicated with a heat colour palette. Row side colours correspond to infection microbial clusters. Column side colours correspond to the taxonomic unit importance. Sample day and infection status are indicated on the right of the heatmap. Numbers correspond to day after the ingestion of bloodmeal; NI, uninfected, IN, infected. Samples with the same name correspond to mosquitoes replicates killed for a given day (days 5, 9 and 14).
the Enterobacteriaceae family (enter5) was higher for infected than for uninfected mosquitoes (P < 0.001). At the genus level, signals of probes targeting Enterobacteriaceae sequences were also higher for CHIKV-infected than for uninfected mosquitoes, including (i) Ecoli1b (P < 0.001) and enter6 (P < 0.001) targeting the Escherichia, Citrobacter, Salmonella and Shigella sequence cluster; (ii) enter3 (P < 0.001) and enter4 (P < 0.001) targeting sequences from other Escherichia species (E. coli and E. albertii); and (iii) Pagg6 targeting the Pantoea agglomerans sequence (P < 0.001).

On the contrary, relative intensities of probes characteristic of Alphaproteobacteria, Bacteroidetes and Planctomycetes decreased with CHIKV infection. These probes include Alpha685 for Alphaproteobacteria (P < 0.01), Blatta2 for Blattabacterium (P < 0.01), Plancto12 for Planctomycetes (P < 0.05), as well as OP11-5 for uncultured Eubacteria WCHB1 (OP11 division) and OP2-1 for phylum OP2 (P < 0.05). Positive probes targeting Alphaproteobacteria sequences that had a weaker signal intensities in CHIKV-infected mosquitoes included (i) Rhodobact1B for bacteria affiliated with the Rhodobacteraceae family (P < 0.01), (ii) Brady4 for Bradyrhizobiaceae family (P < 0.05) and (iii) Wol3 (P < 0.05) and Wol4 (P < 0.01) for the genus Wolbachia.

As Wolbachia is the most prevalent symbiont in Ae. albopictus (Kittayapong et al. 2002; Zouache et al. 2011), we quantified the decrease in relative density of this bacterium by quantitative PCR. Wolbachia relative density was measured as the ratio of wsp wAlbA or wAlbB copy number to host actin copy number (Fig. 5). In mosquitoes fed on uninfected bloodmeal, Wolbachia relative density increased, not significantly, between day 0 and day 13 from $10^{0.98} \pm 10^{0.01}$ to $10^{1.12} \pm 10^{0.05}$ for wAlbA (P < 0.67) (Fig. 5A) and from $10^{0.96} \pm 10^{0.02}$ to $10^{1.18} \pm 10^{0.18}$ for wAlbB (P < 0.08) (Fig. 5B). The infectious CHIKV bloodmeal led to a significant decrease (P < 0.001) in the relative density of each Wolbachia strain. At day 0 post-infection, the relative density of Wolbachia per mosquito was $10^{0.97} \pm 10^{0.02}$ for wAlbA and $10^{0.95} \pm 10^{0.04}$ for wAlbB. Wolbachia density decreased significantly by day 13 post-infection to $10^{0.34} \pm 10^{0.01}$ wAlbA per mosquito (P < 0.001) (Fig. 5A) and $10^{0.41} \pm 10^{0.07}$ wAlbB per mosquito (P < 0.01) (Fig. 5B). These results are consistent with those obtained from microarrays (Fig. 4).

**Change in bacterial community following bloodmeal**

As previously mentioned, microarray data analysed with FF-MANOVA indicated that 38% of the observed variance was explained by the time elapsed after mosquito feeding. The bacterial diversity at days 0, 2, 5, 9 and 14 after the bloodmeal was examined using the Kruskal-Wallis test. Overall, a change in bacterial community structure was observed. Alpha- and Deltaproteobacteria, Bacteroidetes and Firmicutes were positive for both CHIKV-infected and uninfected mosquitoes, contributing significantly to time discrimination (Table 2). The greatest variation in the relative intensity was for probes corresponding to acetic acid bacteria of the Alphaproteobacteria. Indeed, probes Aceto2, Aceto3A and Aceto3B for the Acetobacteraceae family and probes corresponding to genera Acidiphilum (Aci), Acidocella (Acidocella1), Acidomonas (Acidomonas2 and Acdp821), Asia (Asia2) and Glucanacetobacter (Inteur1) increased significantly with time after bloodmeal (Table 2). A similar increase was observed for probes targeting Rhiz-
Table 2 Dynamics of bacterial groups in uninfected and infected mosquitoes after the bloodmeal

<table>
<thead>
<tr>
<th>Phylogenetic affiliation of the target</th>
<th>Target</th>
<th>Probes</th>
<th>Mean relative intensity for infected mosquitoes</th>
<th>Mean relative intensity for uninfected mosquitoes</th>
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<td>D0 D2 D5 D9 D14 P value</td>
<td>D0 D2 D5 D9 D14 P value</td>
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<td>Proto bacteria</td>
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<td>Acetobacteriaceae (excepted Arachnius, Acidiphilum, Acidoceilla, Acidiphilum, Rhodobacter, Rhodococcus, Acidiphilum, Propionibacterium)</td>
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<td>Some Acetobacteriaceae</td>
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<td>Asia</td>
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<tr>
<td>Gluconacetobacter</td>
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<tr>
<td>intermediae and Gluconacetobacter</td>
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<tr>
<td>europus</td>
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<tr>
<td>Anaplasmatenae</td>
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<tr>
<td>Coxiella</td>
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<tr>
<td>Ehrlichia</td>
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<tr>
<td>Bradyrhizobiaceae</td>
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<tr>
<td>Rhizobiaceae</td>
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<tr>
<td>Some Rhizobiaceae</td>
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<td>Mesorhizobiaceae</td>
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<td>Phyllobacterium</td>
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<tr>
<td>Methylobacterium</td>
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<td>Acetobacter</td>
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<tr>
<td>Gluconacetobacter</td>
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<tr>
<td>Rhizobiaceae cluster (including Agrobacterium)</td>
<td>Rhira7</td>
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<tr>
<td>Rhizobiaceae (excepted Agrobacterium, Bradyrhizobiaceae, Brucelina and Brevundimonas)</td>
<td>Rhizo157</td>
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<tr>
<td>Methylbacteriaceae</td>
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<td>Rhodobacteriaceae</td>
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<tr>
<td>(excepted Paracoccus, Amacriococcus, Rhodobacter and Rhodovulum)</td>
<td>Methyo1</td>
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<tr>
<td>Rhodospirillaceae</td>
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<tr>
<td>Azospirillum (excepted A. irakense and A. amanzenese), some Roseomonas spp, some Rhodospirillum spp., some Rhodocista spp., some Skermanella spp.</td>
<td>Azo1</td>
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<tr>
<td>Deltaproteobacteria</td>
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<tr>
<td>Polysphaeraceae</td>
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<tr>
<td>Uncultured Chromomycetes</td>
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obiales sequences. These included probes for the Bradyrhizobiaceae family (Bradhy4), for the Rhizobiaceae family (Rgal157, Rhira7 and Rhizo157) and for the Methylobacterium genus (Methylo1). Azo1 and Azo5 probes for the genus Azospirillum also increased significantly with time. By contrast, a significant reduction in signal intensity was observed for probes targeting sequences from other Alphaproteobacteria such as members of genera Cowdria (cow1) and Ehrlichia (Ehrli), and Rhodobacteraeae family (Rhodobacter1). In the same way, signal intensities for probes indicative of bacteria from the phyla Deltaproteobacteria (probe un43Chon4 for uncultured Chondromycetes), Firmicutes (probes LGC354A and LGC354B, Paeni7-1 and Pbo1 for Paenibacillus) and Bacteroidetes (probe Blatta2 for Blattabacterium) decreased with time (Table 2).

A subcommunity of bacteria (92 positive probes) was not significantly different for all modalities and times (Table S1, Supporting Information). This mostly included bacteria from phyla Proteobacteria (71.7%), that is, Alpha- (21.2%), Beta- (31.8%), Gamma- (36.4%), Delta- (7.6%) and Epsilon Proteobacteria (3%), as well as Firmicutes (12%), Bacteroidetes (5.4%), Actinobacteria (3.3%) and Acidobacteria (4.3%) and Cyanobacteria (3.3%) (Table S1, Supporting Information).

Discussion

There is increasing evidence that interactions occur between resident or introduced microbial taxa in arthropods and invading pathogens (Azambuja et al. 2005; Brownlie & Johnson 2009; Cirimotich et al. 2011; Weiss & Aksoy 2011). For instance, previous studies on Wolbachia indicated that it interacted with CHIKV in mosquitoes (Moreira et al. 2009; Mousson et al. 2010). Using culturing and denaturing gel electrophoresis methods, we previously found that Proteobacteria and Firmicutes dominated the bacterial communities associated with Ae. albopictus from the Indian Ocean, and the bacterial diversity and composition were influenced by the environment inhabited by the mosquitoes (Zouache et al. 2009, 2011). Here, using a taxonomic microarray targeting more diverse bacterial taxa, we showed that the bacterial community in Ae. albopictus ALPROV strain originating from La Reunion island was more diverse than previously described (Zouache et al. 2009) and that various endosymbionts could interact with each other and with CHIKV within the host.

The infection status of mosquitoes was established by quantifying virus and bacterial abundance in individual mosquitoes, making it possible to compare the diversity and composition of the bacterial populations in mosquitoes fed with CHIKV-infected or uninfected blood. Overall, our results revealed that community composition was influenced primarily by the time elapsed after the bloodmeal and secondarily by CHIKV infection. Variations owing to virus infection were significant, although restricted to fewer taxa. The bloodmeal induced a change in the composition of the bacterial community, but not of its structure, whereas the density of bacteria changed.

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s slightly with ageing of mosquitoes, probably due to the modified nutritional conditions (Dillon et al. 2010).

A core bacterial community in *Ae. albopictus*, modified neither by infection nor by the bloodmeal, was mostly represented by *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Acidobacteria*. The presence of core taxa has been noted previously in other environments, and it may function to stabilize the community. When the bacterial community was modified, most changes concerned abundance as infected and uninfected samples differed in terms of probe signal intensity rather than in number of positive probes. Variability in the signal intensity of a given probe is expected to reflect a modification of the number of target rRNA molecules, unless nonspecific binding occurred. For instance, the intensity of specific probes corresponding to *Wolbachia* was shown to decrease in CHIKV-infected but not in uninfected individuals. Quantitative PCR confirmed that, as opposed to uninfected mosquitoes, the relative density of *Wolbachia* in CHIKV-infected mosquitoes decreased as the CHIKV titre increased. Therefore, the differences in probe signal intensities between samples may be interpreted as differences in abundance. The results showing that *Wolbachia* relative density decreases concomitantly with CHIKV replication were consistent with our previous studies (Mousson et al. 2010). The signal intensity of the probe for the *Blattabacterium* was significantly higher in uninfected mosquitoes. This is the first evidence for *Blattabacterium* presence in mosquitoes. As it is one of the bacteria strongly discriminating between infected and uninfected individuals, it should be the focus of further investigation to localize it and to determine its role in insect physiology. Other probes, the intensity of which decreased upon CHIKV infection, also corresponded to phyla *Alphaproteobacteria*, such as members of families *Bradyrhizobiacae* and *Rhodobacteriaceae*, *Bacteroidetes* and *Plantomycetes*.

Conversely, CHIKV invasion resulted in a higher intensity of probes corresponding to *Beta- and Gamma- proteobacteria*. Although no function can be assigned to bacteria identified through *rrs* microarray technology, these bacterial taxa inhabit diverse environments and some are known to establish facultative or mutualistic symbioses with insects (reviewed in Oliver et al. 2010). This metabolic feature may be important for insects to cope with the cost of microbial infection. Interestingly, the intensity of probes targeting sequences from acetic bacteria increased significantly over time for both infected and uninfected individuals. This increase suggests that acidification may accompany blood degradation in mosquitoes. Among acetic bacteria, the genus *Asaia* is known to be associated with *Anopheles stephensi* and *Aedes* mosquitoes where it is vertically transmitted from one generation to the next (Favia et al. 2007; Crotti et al. 2009; Damiani et al. 2010; Zouache et al. 2011).

It is not clear whether CHIKV invasion affects distinct bacterial taxa or whether it is a collateral effect resulting from the *Ae. albopictus* innate immune response to combat viral infection (Sanchez-Vargas et al. 2004, 2009; Sanders et al. 2005; Xi et al. 2008; Fragkoudis et al. 2009). Alternatively, virus infection might compete with the mosquito-associated microbiota for limited resources or space. To corroborate this hypothesis, it would be interesting to localize bacteria after viral infection and bloodmeal and to focus on key organs for viral dissemination/replication and transmission, that is, midgut and salivary glands. Although the over-replication of CHIKV coincides with a decrease in *Wolbachia* relative density in CHIKV-infected individuals, the total bacterial community does not respond in the same way.

Altogether, the results show that while the size of the whole bacterial community remains the same upon virus infection, sizes of subpopulations change. These subpopulations include taxa with probable direct and indirect roles in determining the outcome of infection status. This is the first study to show that virus infection affects the composition and dynamics of total bacterial community in mosquitoes. To date, no treatment or vaccine is available for most arboviruses including CHIKV. The bacterium *Wolbachia* is a candidate to limit the transmission and spread of arboviruses using symbiosis-based control (reviewed in Aksoy 2008). But our results suggest that other bacteria could also be candidates. While there is increasing evidence for both positive and negative effects of natural or introduced bacteria on parasite development and transmission (Azambuja et al. 2005; Dong et al. 2009; Cirimotich et al. 2011; Weiss & Aksoy 2011), our results highlight the importance of considering the whole microbial community and their reciprocal interactions, to better appreciate the phenomenon of interference in determining vector competence. However, as the study is based exclusively on 16S rRNA gene microarray using one *Ae. albopictus* line and one virus strain, the data should be taken in the context that expression of functional bacterial and viral genes and influence of the host genetic background are lacking. This limitation is potentially significant because the type of the interaction may vary depending on all interacting genetic determinants. This line of reasoning is that the extended mosquito phenotype, at the individual level, might result from interactions (cooperation or competition) between communities of genes belonging to different entities (nuclear and symbionts). Multipartite interactions between a community of genetic compo-
ments from hosts and symbionts might also have an impact at population and community levels as they could participate in, for example, local adaptation to changing environment, colonization of new biotopes (invasion), evolution and speciation. Future work on mosquito vector systems should therefore seek to integrate the concept of the extended phenotype, from competence through to adaptive capacity, resulting from interacting genomes, in a community genetics approach (Whitham et al. 2006). This will help in deciphering the mechanisms involved in viral transmission and improve the present use of symbiotic control strategies (Christodoulou 2011).

Acknowledgements

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This study is part of K.Z.’s PhD research devoted in investigating the role of bacterial communities in transmission of arboviruses by mosquitoes. The work was accomplished under supervision of P.M., the head of the team “Microbial Dynamics and Viral Transmission” involved in a broad range of studies on microbial ecology in eukarya, including arthropod vectors such as mosquitoes and ticks, in the unit “Microbial Ecology” at the University of Lyon. R.J.M. was a postdoctoral statistician under supervision of G.L.G., an environmental microbiologist. A-B.F. is head of “Arboviruses and Insect Vectors Laboratory” in the department of Virology at the Institut Pasteur in Paris, working on a wide range of topics regarding the transmission of arboviruses by mosquitoes.

Data accessibility
DRYAD entry:doi:10.5061/dryad.3486n266.

Supporting information
Additional supporting information may be found in the online version of this article.

Table S1 Core of bacterial community of *Aedes albopictus*.

Fig. S1 Determination of the optimal number of probes specific to infection status using the out-of-bag as the minimization criterion in the backward probe selection algorithm based on conditional inference random forest (CI-RF).

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