Prevalence, genomic and metabolic profiles of Acinetobacter and Asaia associated with field-caught Aedes albopictus from Madagascar

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Abstract
The presence of cultivable bacteria Acinetobacter and Asaia was recently demonstrated in the mosquito vector Aedes albopictus. However, it is not known how prevalent these bacteria are in field populations. Here, the presence of these bacteria in Ae. albopictus populations from Madagascar was diagnosed by amplification of 16S rRNA gene fragments. Both genera were detected at relatively high frequencies, 46% for Asaia and 74% for Acinetobacter. The prevalence of Acinetobacter correlated significantly with mosquito gender, and the prevalence of Asaia with the interaction between mosquito gender and the sampling site. For each bacterial genus, more male than female mosquitoes were infected. Using pulse field gel electrophoresis, no significant difference in genome size was found between Acinetobacter isolates from mosquitoes compared with free-living Acinetobacter. However, a great diversity was observed in plasmid numbers (from 1 to 12) and sizes (from < 8 to 690 kb). Mosquito isolates utilized fewer substrates than free-living isolates, but some substrates known as blood or plant components were specifically utilized by mosquito isolates. Therefore it is likely that a specific subpopulation of Acinetobacter is selected by Ae. albopictus. Overall, this study emphasizes the need to gain a global view on the bacterial partners in mosquito vectors.

Introduction
Mosquitoes are hematophagous insects that transmit many pathogens, such as those causing malaria, filariasis and arbovirosis, leading to millions of deaths every year (Christodoulou, 2011). Madagascar and its neighbouring islands have experienced severe epidemics of arboviruses, notably of chikungunya and dengue, and the mosquito species Aedes albopictus was identified as the primary vector responsible for these outbreaks (Ratsitorahina et al., 2008). Aedes albopictus is able to transmit no fewer than 22 different viruses, so there have been many efforts to limit its expansion (Reiter, 1998) mainly by using chemical insecticides. However, negative consequences like the emergence of insecticide-resistant mosquitoes, environmental contamination and the extermination of non-target organisms have called chemical-based methods of control into question (David et al., 2010). Alternative strategies of controlling insect-borne diseases have been proposed based on the use of symbiotic bacteria (Christodoulou, 2011; Hoffmann et al., 2011). For a symbiont to be amenable as a biological control agent in this context, it needs to exhibit a certain level of prevalence, dominance and stability in the insect body (Riehle & Jacobs-Lorena, 2005).

Wolbachia is the most studied bacterial symbiont in Ae. albopictus, but recently two cultivable Acinetobacter and Asaia bacteria were found in this mosquito species (Crotti et al., 2009; Zouache et al., 2011), thus enlarging...
the scope for research into possible biological control strategies. Members of the genus *Asaia* are Alphaproteobacteria able to produce acetic acid (Crotti et al., 2010). The occurrence of *Asaia* was already reported in different insect species including the mosquitoes *Ae. albopictus* and *Aedes aegypti* (Crotti et al., 2009; Chouaia et al., 2010; Damiani et al., 2010). In *Anopheles stephensi*, the Asian malaria mosquito vector, these bacteria are able rapidly to cross tissue barriers and disperse throughout the whole body of insect host (Favia et al., 2007). Members of *Acinetobacter* are hosted by a number of insects (Dillon & Dillon, 2004), but their occurrence in hematophagous groups is poorly documented. Recently, different *Acinetobacter* species were found in natural populations of tsetse fly *Glossina* spp. (Geiger et al., 2009) and *Ae. albopictus* (Zouache et al., 2011). Both *Asaia* and *Acinetobacter* are found in the mosquito organs where viruses replicate (Crotti et al., 2009; Zouache et al., 2009) and they can be cultured and genetically transformed, so it may be possible to exploit these properties to study hematophagous insect-microorganism interactions in more depth.

The objective of this study was to survey the prevalence of *Asaia* and *Acinetobacter* in natural populations of *Ae. albopictus* from Madagascar with respect to insect gender and ecological behaviour. To discover possible bacterial signatures of genomic adaptation, *Acinetobacter* isolates obtained in the survey were compared by plasmid and metabolic profiling.

**Materials and methods**

**Sampling areas and mosquito collection**

The sampling areas and capture procedure were approved by Madagascar National Parks. Mosquito samples were collected in December 2010 at four sites in three regions of Madagascar: Analamanga, Antsinanana and Vatovavy-Fitovivany. Samples that had been collected from another site in the Diana region in December 2009 were also included in the survey. The main characteristics of the sampling sites are given in Table 1. Butterfly netting was used to collect both female and male mosquitoes flying just above grass or the ground. Some laboratory-reared insects that had been collected as pupae 1 year before at Tsimbazaza Park were also studied. *Aedes albopictus* males and females were identified using morphological characteristics keys (Ravaonjanahary, 1978). Only non-blooded mosquitoes were used for the analysis. Most mosquitoes were stored in tubes containing 70% ethanol while the remainder were kept alive and transported to the microbiology laboratory for bacterial isolation.

**Genomic DNA extraction and diagnostic PCR of the rrs gene**

DNA was extracted from 30 male and 30 female mosquitoes selected from the samples collected at each site as previously described (Zouache et al., 2011). PCR amplification of bacterial 16S rRNA-encoding *rrs* genes was done using 30 ng of DNA template in 25 μL of reaction mixture containing 1× buffer (Roche, Switzerland), 40 μM of each deoxynucleoside triphosphate, 0.2 μM each of primers pA and pH (primer details in Supporting Information, Table S1), 0.06 mg mL⁻¹ T4 gene protein 32 (Roche) and 0.028 U of Expand DNA polymerase (Roche, France). Nested PCR was then used to screen for the presence of *Asaia* and *Acinetobacter* DNA in positive PCR products (1 μL). Reactions (25 μL) containing 1× polymerase reaction buffer (Invitrogen, France), 1.5 mM MgCl₂, 40 μM of each deoxynucleoside triphosphate,
0.2 μM of each primer pair (Table S1) and 0.5 U of Taq DNA polymerase (Invitrogen) were carried out in a
T-Gradient Thermocycler (Biometra, France). 

Acinetobacter and Asaia DNA was amplified as previously described
(Crotti et al., 2009; Zouache et al., 2009). For each set of
PCR reactions, bacterial DNA extracts from reference
strains Acinetobacter ATCC 23055 and Asaia ATCC BAA-
21 were used as positive controls. Amplified DNA frag-
ments were separated by electrophoresis through 1% or
1.5% agarose gels stained with ethidium bromide and
visualized under ultraviolet illumination.

Sequencing bacterial rrs and mitochondrial
genomes and phylogenetic analyses

From the diagnostic PCR described above, five positive
PCR products were randomly selected for both male and
female mosquitoes from each bacterial genus and for each
sampling site. Mitochondrial genes encoding cytochrome
oxidase I (COI) and NADH dehydrogenase subunit 5
(NDS) were also sequenced from three males and three
females per site, as previously described (Raharimalala
et al., 2012). PCR products were sequenced at Biofidal-
DTAMB (FR Bio-Environment and Health, Lyon, France)
and the sequences were analysed with theBLASTN pro-
duction at 30 °C –

Plasmid content

Two different methods were used to search for the pres-
ence of plasmids in Acinetobacter. To identify high molec-
ular-weight plasmids, the procedure of Eckhardt (1978)
was used with the modifications previously described
(Mavingui et al., 2002). For this, Acinetobacter strains
were grown in LBM until the optical density of the
culture at 600 nm reached 0.5, then 300 μL of culture

Assays for carbon and nitrogen source
utilization

The Biolog (Hayward, CA) Phenotype MicroArrays (PM)
system and the Omnilog reader (Hayward, CA) were
used to study the metabolic profiles of the different
Acinetobacter isolates and reference strains (Table S2).
Standard commercial microplates (PM1–3) were used to
simultaneously test for the ability of the microorganism
to oxidize a panel of 190 different carbon sources (PM1
and PM2) and 95 different nitrogen sources (PM3),
following the manufacturer’s standard operating condi-
tions. The PM plates were placed in an aerobic OmniLog
incubator at 28 °C to collect signal data every 15 min for
48 h. The signal data (conversion of pixel density to a
signal value reflecting cell growth) were analysed using
BIOLOG MICRO-LOG3 4.20 software (Biolog, Inc.) and
the average signal value for each well was exported to
Microsoft Office Excel (Microsoft) to be compiled. Sub-
strate utilization was scored as positive when its growth
value was at least 20% of the growth value recorded on
d-alanine, a substrate used by all the strains (i.e. growth
value of 100%).

Enrichment, isolation and identification of
Acinetobacter

Field-caught live mosquito specimens were anaesthetized
with ether. Six adult mosquitoes per site (three males and
three females) were surface-disinfected and crushed indi-

separately in 150 μL sterile 0.8% NaCl. Homogenates
(10 μL) were streaked directly on plates containing a
modified rich solid Luria–Bertani medium containing
NaCl at 5 mg mL−1 (LBM) then incubated at 26 °C.
Homogenates (20 μL) were also added to 1 mL of enrich-
ment medium (0.2% KNO3, 0.02% MgSO4·7H2O, 0.2% 
sodium acetate, 0.04 M KH2PO4, pH 6.0; Bouvet & Joly-
Guillou, 2000) and incubated at 30 °C for 24–48 h with
shaking. When microbial growth occurred, an 10-
aliquot of the culture was streaked onto Herellea agar

plates (Biolife, Italy; Mandel et al., 1964) and incubated
at 37 °C for 24–48 h. Single distinct colonies of different
colours or shapes were selected and re-inoculated onto
fresh agar plates of the corresponding medium. Resultant
colonies were streaked out again to check for purity and
samples were stored in 25% glycerol at −80 °C. All iso-
lates were screened by colony PCR amplification of the
rrs gene using specific primers for Acinetobacter. For this,
a single colony was suspended in 25 μL sterile water and
boiled for 2 min, then placed on ice for 2 min. The boil-
ing and cooling steps were repeated followed by centrifug-
ation at 16 100 g for 5 min. Each 50-μL reaction
contained 2 μL supernatant, 1× PCR buffer, 2 mM
MgCl2, 0.5 mM dNTP, 0.5 μM of each primer Acin1 and
Ac (Table S1) and 1 U Taq Polymerase (Invitrogen). PCR
conditions, sequencing of positive PCR products and
analysis were as described above. Complete rrs genes of
Acinetobacter isolates were amplified by colony PCR using
the universal bacterial primers pA and pH (Table S1).

from Madagascar


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was analysed. Electrophoresis was carried out at 10 V for 20 min and 85 V for 210 min. To identify lower molecular-weight plasmids, a second method described by Seifert et al. (1994) was used. The size of a plasmid was estimated by comparing its mobility in agarose gels to the mobility of plasmids from sequenced Azospirillum genomes (Caballero-Mellado et al., 1999; Wisniewski-Dyé et al., 2011) and standard supercoiled plasmids (Life Technologies, Inc.).

**Pulse field gel electrophoresis**

The undigested genomes of *Acinetobacter* isolates were analysed by pulse field gel electrophoresis (PFGE) according to published protocols with some modifications (Schwartz & Cantor, 1984). Briefly, isolates were grown in LBm liquid medium for 18 h at 30 °C. Bacteria were subcultured to ensure they were pure. Cell cultures were centrifuged at 5000 g for 20 min at 4 °C. The pellet was washed once and resuspended in 1× Tris-EDTA buffer. The optical density of the suspension at 600 nm was adjusted to 1.8 and 2.0. An equal volume of a 1.6% low melting-point agarose (Bio-Rad) was mixed with cell suspensions and 150 μL of the mixture was placed in a plug mould (Bio-Rad). Cells were lysed using a lysis solution (2× Tris NaCl EDTA, 10% sodium lauroyl sarcosinate, 1.4 mg mL⁻¹ lysozyme) at 37 °C for 24 h, then proteins digested with 0.5 mg mL⁻¹ proteinase K (Euromedex) in 0.5 M EDTA pH 8 and 1% lauryl-sarcosine at 37 °C for 48 h. Electrophoresis was carried out in 0.8% chromosomal-grade agarose in 1× TAE buffer using a CHEF Mapper XA (Biorad, France) at 14 °C, a constant pulse of 500 ms and a field angle of 106° for 48 h at 3 V cm⁻¹.

**Statistical analysis**

To investigate the effect of sampling site and mosquito gender on the prevalence of each bacterial genus in the field populations, logistic regression models were built considering all possible combinations of additive effects and interactions. The most appropriate model was selected for each type of bacterium using the Akaike information criterion (AICc) adjusted for small sample size (Anderson et al., 1994). The models were ranked according to the smallest AICc differences (denoted ΔAICc) between the focal model and the lowest AICc model. When ΔAICc was larger than 2, the model with the smallest AICc was selected. Parameter estimates were obtained by the method of maximum likelihood. Possible interactions between both bacteria were sought using the corrected chi-squared method (Hellard et al., 2012). To take account of factors that could potentially create statistical associations (i.e. false interactions), both identified risk factors for the two bacteria (i.e. the mosquito sex and the sampling site) were included in the logistic regression model to estimate the expected frequencies. All analyses were performed in R software (R Development Core Team, 2009).

**Nucleotide sequence accession numbers**

The COI and ND5 gene sequences were submitted to GenBank under accession numbers JN406664–JN406666, JN406668–JN406670, JN406694, JN406697, JN406700–JN406705, JN406709–JN406711, JN406714, JN406715, JN406727–JN406732, JN406738–JN406740, JN406742–JN406744, JN406763–JN406767, JN406771–JN406776, JN406779–JN406783, JN406789–JN406794, JN406804–JN406809, and JN406840–JN406844. The rRNA gene sequences obtained from direct PCR amplification (JN379293–JN379351) and from *Acinetobacter* isolates (JQ408696–JQ408701 and JQ627634) are also available in GenBank.

**Results**

**Prevalence of *Acinetobacter* and *Asaia* in *Ae. albopictus***

Alignment of all COI and ND5 gene sequences of *Ae. albopictus* showed polymorphism of only 0.01% and 0.004%, respectively (not shown), revealing a strong phylogenetic relationship between mosquito populations from the five sampling sites. Diagnostic PCR amplification of the 16S rRNA-encoding *rrs* gene was used to assess the prevalence of *Acinetobacter* and *Asaia* in field and laboratory-reared populations of *Ae. albopictus*. The overall infection frequencies were quite similar between field and laboratory-reared populations (Table 2) with a higher infection frequency

### Table 2. Prevalence of *Acinetobacter* and *Asaia* in *Aedes albopictus*

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acinetobacter</td>
<td>Asaia</td>
<td>Acinetobacter</td>
</tr>
<tr>
<td>Laboratory populations</td>
<td>70% (35/50)</td>
<td>46% (23/50)</td>
<td>59% (19/32)</td>
</tr>
<tr>
<td>Field populations</td>
<td>74% (184/249)</td>
<td>46% (117/252)</td>
<td>79% (96/121)</td>
</tr>
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</table>

Number of infected mosquito individuals vs. number of mosquito individuals analysed is indicated in parentheses.
for *Acinetobacter* (around 70%) than for *Asaia* (46%). Infection frequencies were compared between field populations sampled in the same year, i.e. data from specimens from the Diego site in the Diana region were excluded from the statistical analysis. According to the AICc, the best logistic regression model to explain *Acinetobacter* prevalence included the gender effect (Table S3), which is in keeping with the statistical analysis showing a significant difference in infection frequencies according to the sex of the mosquito. Males are two times more infected than females (*P* = 0.04, Table S3), contrary to what was observed in laboratory populations. For *Asaia*, the selected model included the interaction between the sex and the sampling site (*P* = 3.84 × 10⁻¹¹; Table S3). Males tended to be more infected than females except in Mananjary, where the opposite was true (Table S4). When the identified risk factors (i.e. mosquito sex and sampling site) were taken into account, a probable interaction between the two bacteria was also revealed ($\chi^2_{corr} = 6.45124, P = 0.001$). Double-infected mosquitoes account for 50% (119/237) of the total caught, with proportionally more double-infection of females (54%) than of males (47%). This interaction seems to be synergistic because more double-infected mosquitoes were observed than would be expected if the bacteria acted independently.

The partial sequences of *rrs* genes amplified from the *Acinetobacter*-positive mosquito samples allowed them to be presumptively assigned to seven species with percentages of identity ranging from 96% to 100%: *Acinetobacter baumannii*, *Acinetobacter junii*, *Acinetobacter johnsonii*, *Acinetobacter schindleri*, *Acinetobacter calcoaceticus* and *Acinetobacter lwofi*. A phylogenetic tree clusters the sequences of the different species of *Acinetobacter* with those obtained after colony isolation and those from corresponding reference strains (Fig. 1).

**Molecular characterization of *Acinetobacter* isolates from *Ae. albopictus***

Attempts were made to culture bacteria from *Ae. albopictus* mosquito samples. Diagnostic PCR allowed to identify four isolates potentially affiliated with *Acinetobacter*. On the basis of the nearly complete *rrs* gene sequences obtained (1409–1467 bp long), two isolates cultured on Herellea agar plates were judged to be affiliated to *A. baumannii* and two isolates cultured on LBm to *A. lwofi*. To further characterize these isolates from mosquitoes, they were compared to five additional strains: one from *A. calcoaceticus*, two from *A. johnsonii* that had been isolated from *Ae. albopictus* in our previous study (Zouache *et al.*, 2011), and two other *Acinetobacter* sp. isolates recovered from water in which *Ae. albopictus* larvae were breeding in the laboratory (Table S2).

PFGE was used to analyse the genetic material of isolates. All isolates contained a high molecular-weight replicon (c. 3 Mb) which considered alongside sequenced reference genomes probably corresponds to the bacterial chromosome (Fig. 2). A second replicon (c. 1 Mb) is also seen in the PFGE pattern from mosquito isolates 1b, 3a and 8 and reference strains Ref-1, Ref-3 and Ref-4. Intraspecific comparisons showed that mosquito isolates of *A. lwofi* (2a and 2b) and *A. calcoaceticus* (3a) had a similar pattern to one of their corresponding reference strains. For *Acinetobacter baumannii*, only one of the two mosquito isolates (1b) had a similar PFGE pattern to that of the reference strain. By contrast, the PFGE patterns produced from mosquito isolates of *A. johnsonii* (4a and 4b) were similar to each other yet distinct from their reference strain. Isolates 8 and 9 from medium in which laboratory mosquitoes had been bred had PFGE profiles closely related to that of the *A. johnsonii* reference strain. A great diversity in both the number and size of plasmids was observed both between isolates and between isolates and their respective reference strains (Fig. 3). With the Eckhardt procedure for lysis and plasmid extraction it was possible to detect low molecular-weight (up to 75 kb) and high molecular-weight (up to 690 kb) plasmids (Fig. 3). The mosquito isolates of *A. lwofi* contained the most plasmids (> 12 replicons).

**Metabolic characteristics of *Acinetobacter* isolates**

The metabolic capabilities of *Acinetobacter* isolates were explored using BIOLOG assays. Two independent experiments were done giving similar results. The bacterial strains were found to have different capacities for substrate utilization with both broad and narrow spectra suggesting variability in endogenous enzymatic activities (Table S5). The reference strains tend to utilize a broader spectrum of substrates than isolates from mosquitoes: 36.3% vs. 28.5% for *A. calcoaceticus*, 20.7% vs. 18.8% for *A. johnsonii*, 22.2% vs. 15.1% for *A. lwofi* and 28.5% vs. 29.1% for *A. baumannii*, respectively. At the intra-species level, some substrates were specifically used by reference strains whereas others were catabolized only by mosquito isolates (Table 3).

**Discussion**

This study provides an estimate of the prevalence of *Acinetobacter* and *Asaia* in natural populations of the mosquito *Ae. albopictus* in Madagascar. The overall infection frequency of *Acinetobacter* (c. 70%) was higher than that of *Asaia* (c. 46%). High prevalence is one of the criteria that may indicate there is an intimate symbiotic
relationship between a given bacterium and its host. Previous studies demonstrated that the \textit{Asaia} genus was 100\% prevalent in laboratory populations of \textit{Ae. albopictus} (Chouaia \textit{et al.}, 2010). Here we report the much lower prevalence of \textit{Asaia} in natural populations of \textit{Ae. albopictus} from Madagascar. This difference suggests that environmental factors strongly influence prevalence. Members of the genus \textit{Asaia} are widely distributed in nectar-bearing flowers (Yamada & Yukphan, 2008), whereas \textit{Acinetobacter} are mostly free-living saprophytes.
Fig. 2. PFGE of undigested genomic DNA of *Acinetobacter* mosquito isolates and their reference strains. Chromosomal DNA from *Hansenula wingei* (M) was used as a reference (BioRad). Characteristics of the samples are given in Table S2.

Fig. 3. Electrophoretic profiles of plasmids from *Acinetobacter*. Characteristics of the samples are given in Table S2. (a) Electrophoretic profiles of high molecular-weight plasmids obtained by a modified Eckhardt procedure. Plasmids from *Azospirillum brasilense* strains En-Ab79 and Sp245 were used as references (Caballero-Mellado et al., 1999; Wisniewski-Dyé et al., 2011). (b) Electrophoretic profiles of low molecular-weight plasmids obtained by the procedure of Seifert et al. (1994). Standard supercoiled plasmids (Life Technologies, Inc.) were used as references (M). Photograph quality was improved using ADOBE PHOTOSHOP ELEMENTS 6.0 (Adobe).
often present in the rhizosphere, in water, or on the skin of vertebrates (Doughari et al., 2011). Interestingly, these ecological niches can also all be used by mosquitoes as food sources or for laying eggs, hatching larvae, or resting. Sharing such environments may thus favour encounters between mosquitoes and these bacteria. Sex effects on the composition and diversity of mosquito-associated bacteria have already been reported in natural populations of Ae. albopictus (Zouache et al., 2011). Both intrinsic genetic characteristics and ecological behaviour may account for such differences. In particular, only females are hematophagous. Previous studies reported that members of Asaia and Acinetobacter may cause opportunist infections in humans (Alauzet et al., 2010; Doughari et al., 2011), raising the possibility that cross-infection could occur when mosquitoes bite hosts. In addition, females scatter over a wider area than males when looking for a breeding site, increasing the chance of encountering these bacteria. Our findings revealed a possible synergy between the two types of bacteria, although we cannot exclude the possibility that they share an unknown risk factor that could create a statistical association between the bacteria or that some mosquito individuals are particularly sensitive to multiple infections.

The high prevalence of Acinetobacter found in our study raises questions as to how the bacterium is acquired and transmitted. Using fluorescent in situ hybridization, Acinetobacter has been localized in the midgut but not in the ovaries of Ae. albopictus (Zouache et al., 2009), so the bacteria are likely to be acquired from the environment. However, this does not exclude the possibility of vertical transmission by other mechanisms such as coprophagy, paternal transmission or egg-smearing (Kikuchi, 2009). Experimental infection showed that Asaia could be transmitted both vertically and horizontally from parents to offspring (Crotti et al., 2009). Surprisingly, the Asaia infection prevalence reported here does not strongly support the assumption of a high vertical transmission rate. On the contrary, the high prevalence of Acinetobacter suggests efficient transmission and maintenance within host populations, indicating a possible role in the biology of the mosquito in nature. As yet, the role of Acinetobacter in insect biology has only been demonstrated in Stomoxys calcitrans, which need these bacteria to ensure complete

### Table 3. Differential substrate utilization between Acinetobacter mosquito isolates and their reference strains

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>D-galactose, D-mannose, D-melibiose, D-xylose, glucuronamide, β-D-allose, D-fucose, gentiobiose, D-glucosamine, D-hydroxyacetone, D-saccharic acid, mucic acid dextrin</th>
<th>2-o-glucose</th>
<th>2-arabinose, 2-l-lactamide</th>
<th>2-arabinose, 2-xylene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylates</td>
<td>Formic acid, oxalomalic acid, β-D-mannose, β-D-ribose, β-D-glucose, β-D-galactose, α-D-glucose, γ-D-glucose, 2-keto valeric acid, β-D-ribulose, 2-ketoheptonic acid</td>
<td>β-malic acid, 2-ketovaleric acid, 2-ketocitric acid</td>
<td>2-amino butyric acid</td>
<td>Mono-methylsuccinate, β-hydroxy-butyric acid, acetoacetic acid</td>
</tr>
<tr>
<td>Amino acids</td>
<td>2- aspartic acid, glycy1- aspartic acid, glycy1-glutamic acid, l-threonine, l-alanyl-glycy1, l-proline, l-isoleucine, l-leucine, l-valine, alanine-asparagine, alanine-glutamine, alanine-glycy1, glycine-asparagine, glycine-glutamine, glycine-methionine, alanine-threonine</td>
<td>2-isoleucine, 2-ornithine, glycine, 2-asparagine</td>
<td>2-aspartic acid, 2-glutamic acid, 2-pyrroglutamic acid, 2-citrulline, l-L-α-amino-N-butryc acid, 2-α-amino-N-caprylic acid</td>
<td>2-α-amino-N-valeric acid, alanine-glutamine, methionine-alanine</td>
</tr>
<tr>
<td>Alcohol</td>
<td>2,3-butanediol</td>
<td>Formamide</td>
<td>Methylamine</td>
<td>Nitrate</td>
</tr>
<tr>
<td>Amine</td>
<td>Ethanolamine</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nitrogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>Adenine, guanine, xanthine, inosine</td>
<td>Biuret guanosine, xanthine</td>
<td>Biuret, guanine, guanosine, uracil</td>
<td></td>
</tr>
</tbody>
</table>

Not underlined, substrates only used by the reference strain. Underlined, substrates only used by Acinetobacter mosquito isolates.
development of the fly larvae (Lysyk et al., 1999). It has already been shown that some Acinetobacter species are efficient pollutant degraders (Lal & Khanna, 1996). As Ae. albopictus is able to invade suburban areas and survive in polluted zones (Mousson et al., 2005), it is possible that Acinetobacter-associated mosquitoes have an improved capacity to colonize and adapt to anthropized environments. A potential role for Asaia in the biosynthesis of vitamins in the mosquito species Anopheles stephensi was suggested by Crotti et al. (2009).

To gain more insight into the mosquito–bacteria interactions, the genetic contents and metabolic capabilities of Acinetobacter mosquito isolates and their free-living relatives were compared. Despite considerable effort, only a few Acinetobacter were isolated from field-caught Ae. albopictus, contradicting somewhat the high prevalence of Acinetobacter detected by molecular methods. The seven Acinetobacter isolated from Ae. albopictus were phylogenetically closely related to the species A. calcoaceticus, A. baumannii, A. lwoffii and A. johnsonii. The estimated genome sizes of Acinetobacter isolates (c. 3 Mb) were similar to those of Acinetobacter strains reported in published databases ranging from 3.59 to 4.05 Mb (Fondi et al., 2010). It is well known that plasmids are among the most important elements in the evolution of prokaryotes and their adaptation to fluctuating environmental conditions (Eberhard, 1990). The presence and wide diversity of plasmids in Acinetobacter isolates may suggest these genetic elements have a biological role. The functions of many plasmids of Acinetobacter isolates have yet to be identified but some have been associated with metabolic activities or metal resistance (Winstanley et al., 1987; Schembri et al., 1994; Mengoni et al., 2007). Information on phenotypic traits is a useful complement to genomic data (Bochner et al., 2010), so substrate utilization assays were performed to investigate whether the variation observed at the DNA level also reflected variation in the metabolic potential of Acinetobacter isolates. Fairly large metabolic differences were found between the Acinetobacter isolates, and this may related to their phylogenetic relationships given that the nine isolates belonged to distinct genotypes (Geiger et al., 2011). Some Acinetobacter isolates from mosquitoes were unable to utilize certain substrates metabolized by the reference strains, suggesting that substantial changes in gene content or regulation or both had occurred. Conversely, some substrates were only used by mosquito-associated strains. For example, amino acids α-keto valeric acid and glycine, which are blood components, were only metabolized by the isolates of A. baumannii from mosquitoes, whereas 4-hydroxybenzoic acid and xylose, which are common constituents of plants, were only metabolized by A. johnsonii isolates from mosquitoes. Possibly these bacterial isolates improve blood digestion and nectar assimilation respectively by the insect host. Using this approach, a phenotypic or genotypic subpopulation of Acinetobacter from mosquitoes appears to be selected from the surrounding environment.

In conclusion, this study demonstrated the relatively high occurrence of Acinetobacter and Asaia within the mosquito Ae. albopictus. This bacterial prevalence significantly correlated with the sex of the mosquito and environmental factors, notably the climate and ecology. The variability observed emphasizes the need to gain a global view of the diversity and genetic potential of bacterial partners in mosquito vectors.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Primers used in this study.

**Table S2.** Characteristics of *Acinetobacter* isolates and reference strains used in this study.

**Table S3.** Best logistic regression models for *Acinetobacter* and *Asaia* prevalence data according to Akaike information criterion (AICc).

**Table S4.** Final logistic-regression models of mosquito infection risk factors for the two bacteria: variables and variable categories.

**Table S5.** Substrate utilization profiles of the *Acinetobacter* mosquito isolates and their reference strains.

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