RESEARCH ARTICLE

Midgut bacterial dynamics in *Aedes aegypti*

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Abstract

In vector mosquitoes, the presence of midgut bacteria may affect the ability to transmit pathogens. We have used a laboratory colony of *Aedes aegypti* as a model for bacterial interspecies competition and show that after a blood meal, the number of species (culturable on Luria–Bertani agar) that coexist in the midgut is low and that about 40% of the females do not harbor any cultivable bacteria. We isolated species belonging to the genera *Bacillus*, *Elizabethkingia*, *Enterococcus*, *Klebsiella*, *Pantoea*, *Serratia*, and *Sphingomonas*, and we also determined their growth rates, antibiotic resistance, and *ex vivo* inhibition of each other. To investigate the possible existence of coadaptation between midgut bacteria and their host, we fed *Ae. aegypti* cohorts with gut bacteria from human, a frog, and two mosquito species and followed the bacterial population growth over time. The dynamics of the different species suggests coadaptation between host and bacteria, and interestingly, we found that *Pantoea stewartii* isolated from *Ae. aegypti* survive better in *Ae. aegypti* as compared to *P. stewartii* isolated from the malaria mosquito *Anopheles gambiae*.

Introduction

Bacteria living in the gut of insects are affected by the environmental conditions created by the host organism and other microorganisms present. In contrast to higher eukaryotes, insect has a less diverse gut flora (Weiss & Aksoy, 2011). Dillon & Dillon (2004) state that the diversity of the microbial communities is influenced by the presence of specialized gut structures and the effect of pH, redox conditions, digestive enzymes present, and the type of food ingested. The bacteria thus have to adapt to all these conditions, and because some bacteria are beneficial for the host in terms of degradation of food or helping to suppress harmful bacteria, an element of coadaptation can be expected between the bacteria and the host they inhabit. Through evolution, certain bacterial species have developed such close relationship with their insect hosts that they now lack certain functions that would enable them to live outside their host or the host to survive without them. Well-studied examples include

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the microbiota in termites (Abe *et al.*, 2000) and the genus *Buchnera* in aphids (Douglas, 1998). Several species of blood-sucking insects, which are important vectors of disease, also harbor obligate midgut bacterial symbionts. Examples include tsetse flies that carry the symbiont *Wigglesworthia glossinidia* (Aksoy, 1995) and the vector for Chagas disease, the kissing bug *Rhodnius prolixus* that carry *Rhodococcus rhodnii* (Beard *et al.*, 1992). In mosquitoes, obligate symbionts have not yet been found, but a strong symbiotic relationship exists between *Anopheles* mosquitoes and bacteria of the genus *Asaia* (Favia *et al.*, 2007; Damiani *et al.*, 2010).

One of the key species for pathogen transmission is *Aedes aegypti*, which is the main vector of yellow fever and dengue fever. It was described as a vector of yellow fever as early as 1881 (Chaves-Carballo, 2005) and for dengue fever in the early 20th century (Graham, 1903; Bancroft, 1906). Investigations of the *Ae. aegypti* gut flora include species identification (Micks & Ferguson, 1963; Wistreich & Chao, 1963; Gusmão *et al.*, 2007, 2011; Chouaia *et al.*, 2010; Zouache *et al.*, 2011), localization in the gut (Micks *et al.*, 1961; Crotti *et al.*, 2009; Gusmão *et al.*, 2011), survival of gut bacteria (St. John *et al.*, 1930) and impact of gut bacteria on blood digestion and fecundity (Gaio Ade *et al.*, 2011).

Investigating gut flora in mosquitoes can in principle be performed in two different ways, either by collecting wild mosquitoes or by rearing mosquitoes in the laboratory. While catching mosquitoes in the wild gives a true representation of what bacteria they encounter in their natural environment, laboratory studies enable manipulation of the conditions. In that perspective, *Ae. aegypti* is a suitable model for the study of gut flora because of its relative easiness to grow in the laboratory and high relevance for pathogen transmission. To further understand the relation between *Ae. aegypti* mosquitoes and their gut flora, we isolated bacteria from an *Ae. aegypti* colony and investigated their properties; in addition, we infected *Ae. aegypti* of both sexes with bacterial species isolated from different host species and followed the dynamics of the different species in the host over time.

Materials and methods

Mosquito rearing

Aedes aegypti (Rockefeller) mosquitoes were reared at 27 ± 2 °C and 70 ± 10% relative humidity with a 12-h light/12-h dark cycle. Adults were kept in cages made of cardboard boxes with netting on top. The mosquitoes were offered a 10% boiled sucrose–water solution on a soaked Kleenex tissue *ad libitum* and blood from a human volunteer once a week. Eggs were laid on wet filter papers and transferred to water trays with clean tap water in which the larvae then developed. Larvae were fed Tetramin® fish food.

Growth of bacteria after blood meal and analysis of colony morphologies

Living mosquitoes were anesthetized with ether and the exoskeleton washed in ethanol followed by two consecutive baths of water for approximately 5 s each. The mosquito abdomen was then ground in 100 μL 0.9% NaCl. To determine the number of bacteria at a certain time point after a blood meal, the gut-saline preparation of each mosquito was used in a dilution series of undiluted, 10^2, 10^4, 10^6, and 10^8 times dilution in 0.9% NaCl. Each dilution was spread on two Trypticase soy agar plates supplemented with 5% sheep blood (Becton Dickinson Microbiology, France; in total 10 plates per mosquito from a total of 94 mosquitoes, i.e. 940 plates) and incubated at 30 °C overnight. From the dilution series, the dilution that had plates with between 10 and 500 colonies was counted and the number of CFU in the original solution calculated.

Correspondence between morphology and species determination was confirmed purifying a representative of each morphology and then analyzing the isolates by temporal temperature gradient gel electrophoresis (TTGE). The TTGE method separates DNA fragments according to sequence, and the distance migrated by a certain band can therefore be said to correspond to one particular species of bacteria. In comparison with the temperature gradient gel electrophoresis (TGGE), TTGE has an increased sensitivity as the separation range expands because of a uniform and gradual temperature increase over time (Yoshino *et al.*, 1991). In this study, we followed the settings in Lindh *et al.* (2008). In brief, a region of the 16S rRNA gene (about 400 bp) of the bacterial isolates was amplified with PCR primers 968GC f (5′-*CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC GGGGGG*AACGCGAAGAACCTTAC-3′) and 1401 r (5′-C GGTGTGTACAAGACCC-3′). A GC-clamp was attached to one of the primers (968GC f, in italics) to prevent complete strand dissociation during electrophoresis. The PCR was run at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 58–48 °C for 30 s (temperature decreased by 1 °C every cycle for 10 cycles and then held at 48 °C for 20 cycles), 72 °C for 1 min followed by a final extension step at 72 °C for 5 min (Lindh *et al.*, 2005). TTGE conditions were as follows: a 7% polyacrylamide gel containing 8 M urea was run at 70 V in 1.25× TAE buffer starting at 60 °C and raised to 70 °C during 16.5 h ($\sim$0.6 °C h^{-1}). The gels were stained for 10 min with ethidium bromide (1 mg mL^{-1}, 1.25× TAE buffer), rinsed for 10 min

(1.25× TAE), and then visualized on a UV transillumination table.

Determination of properties of isolated bacteria

Isolates were selected based on morphology. Cloning, sequencing, and sequence analysis were performed as previously described (Lindh *et al.*, 2005). In brief, the 16S rRNA gene from the selected isolates was amplified by PCR using forward primer 8f (5′-AGAGTTTGATIIT GGCTCAG-3′, I = inosine) and reverse primer 1501r (5′-CGGITACCTTGTTACGAC-3′). PCR products were cloned into TOPO 2.1 vectors utilizing TA-cloning (Invitrogen) and sequenced at Macrogen, South Korea, on an ABI 3700 automatic DNA sequencer with the M13F and M13R primers corresponding to the TOPO 2.1 sequences. The 16S rRNA gene sequences were analyzed in BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/). To avoid inclusion of chimeric sequences, the Ribosomal Database Project II Chimera Check was used (http://wdcm.nig.ac.jp/RDP/html/analyses.html).

Antibiotic resistance was determined by minimal inhibitory concentration experiments. 25 μL of an overnight culture of the bacteria was added to tubes with 5 mL of Luria–Bertani broth (LB) containing 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 μg mL^{-1} antibiotics, respectively. The tubes were incubated overnight at 30 °C at 160 r.p.m. after which growth was determined visually. Generation times for the bacterial strains were determined in LB at 30 °C during shaking at 160 r.p.m. Optical density (600 nm) was determined by spectrophotometric readings at 1-h intervals. The generation time (doubling time) was calculated from the slope generated during the logarithmic phase.

Bacterial interspecies growth inhibition

To determine whether any of the isolated bacteria could inhibit the growth of the others, 2.5 mL of 1.5% soft agar was inoculated with 250 μL of an overnight culture of one of the bacterial species. The soft agar was then poured onto plates with LB agar and left to solidify. A replicator with 25 spikes was dipped in overnight cultures of the other bacterial species in Bertani trays and then stamped on the soft-agar plate. The plates were incubated at 30 °C overnight and then examined for growth inhibition.

Dynamics of gut bacteria in mosquito cohorts

Five isolates of gut bacteria were used to compare dynamics based on host origin: *P. stewartii* from *Ae. aegypti* isolated in this study (JL2, EF189920) in the paper called PsAa; *P. stewartii* (EF189919) from an *Anopheles gambiae* female field-caught in Kilifi, Kenya, in 1998 [also used in Lindh *et al.* (2008), PsAg]; *Enterobacter agglomerans* from *An. gambiae* kindly provided by J.C. Beier [(Straif *et al.*, 1998), EaAg]; a laboratory strain of the human gut bacteria *Escherichia coli*, EcHs; and *Klebsiella oxytoca* isolated from the gut of the Oriental fire-bellied toad (*Bombina orientalis*) kindly provided by H.G. Boman, KoBo. The bacterial cells of all species were made electrocompetent for plasmid transformation using a standard protocol (Ausubel *et al.*, 2001) and then transformed by electroporation with a GFP-expressing plasmid containing a gene for ampicillin resistance (pGFPuv, Clontech). 30 mL of LB was inoculated with bacteria from an overnight culture; these bacteria were grown at 30 °C, 160 r.p.m. to $OD_{600\ nm} \sim 0.5$. The culture was centrifuged and washed once with 10% sugar solution after which the bacteria were suspended in 10% sugar solution to a final concentration of approximately 10^3 bacteria mL^{-1} and offered to the mosquitoes at day 0. From day 1 and onwards, only sterile sugar solution was given to the colony, and the sugar solution was changed everyday. The sugar solutions (with or without bacteria) did not contain antibiotics. For each species of bacteria, a cohort of 100 mosquitoes were used (50 males and 50 females). From the cohort, 10 males and 10 females were analyzed after 24-h access to bacteria-baited sugar solution (time point called day 1), and then five males and five females were analyzed at days 3, 7, 10, and 15 or 16. Living mosquitoes were anesthetized with ether and the exoskeleton washed in ethanol followed by two consecutive baths of water for approximately 5 s each. The mosquito abdomen was then ground in 0.9% NaCl and diluted into a gut-saline preparation. The gut-saline preparation of each mosquito was diluted three times and plated on LA-plates (in total 281 mosquitoes and 843 plates). Colonies were screened for GFP expression under UV light.

To test for the effect of bacterial species, host sex, and time on bacterial dynamics in the gut of the sampled mosquitoes, we used two separate approaches. Because the data set contained a large amount of zero observations, we first modeled how the probability of bacterial survival in the gut changes over time. Thus, the presence/absence of midgut bacteria was modeled as a function of bacterial species (five levels), host sex (two levels), and time (days after infection, assumed continuous) as well as the interactions between the main effects. The response variable (presence or absence) has a binomial distribution, and thus, we constructed the model with binomial error distribution and logit link function as implemented in the *glm* procedure in R (R Development Core Team,

2008). Second, to the subset of the data containing non-zero bacterial counts, we modeled the number of bacteria as a function of bacterial species, host sex, and time using the *lm* procedure in R. Because the distribution of the data was highly skewed, we log-transformed the data prior to analyses. For both models, the bacterial species *P. stewartii* isolated from *Ae. aegypti* was used as a baseline to which the other bacterial species were compared. This also applies to the effect of host sex where female host was used as the baseline. Thus, the analysis does not estimate parameters for the baseline main effects or any of their interaction effects (Tables 4 and 6). Because initial exploration of the data indicated significant two- and three-way interactions between some of the explanatory variables, the input data (bacterial species, sex, and day) were centered to enable interpretation of the main effects and lower-order interactions (Schielzeth, 2010).

Results

Number of bacteria and morphologies after blood feeding

The development of the gut community after a blood meal in terms of number of bacteria was analyzed for a total of 94 mosquitoes (Table 1). The number of bacteria increased with time by orders of magnitude, and already at 6 h, the majority of bacteria-harboring mosquitoes had more than 10 000 TSA-culturable bacteria in the gut. However, only about 60% of the mosquitoes harbored culturable bacteria.

The development of the bacterial community also includes the number of different species present in the gut. The competition of species in the gut could lead to one particular species taking over in the gut. Therefore, we analyzed number of colony morphologies serving as a proxy for different species for a subset (46) of the mosquitoes and found that of 29 mosquitoes that had culturable bacteria, 23 had one species, three mosquitoes had two species, and three mosquitoes had three species supporting the hypothesis of certain bacterial species being able to exclude or outgrow other species. Of course, we cannot rule out that there might be bacteria that survive in the mosquito gut, but not on plates.

Properties of isolated bacteria

From the initial experiments, eight isolates were selected for further characterization (Table 2). Several isolates have close similarity to other isolates found in mosquitoes. JL1 is a relative to a species of *Elizabethkingia* found in *An. gambiae* (Lindh *et al.*, 2008), which was described

Table 1. Number of blood-fed mosquitoes that contain a certain amount of bacteria

	Number of bacteria					
Time point (p.b.m.)	0	10^0–10^2	$> 10^2$–10^4	$> 10^4$–10^6	$> 10^6$–10^8	% Harboring bacteria
0 h	3	2	1	2		62
3 h	6	1	3	3		54
6 h	13		7	15	5	68
24 h	15			2	16	55

p.b.m., post-blood meal.

Table 2. Properties of bacteria isolated from the *Aedes aegypti* colony

			Antibiotic resistance (μg mL^{-1})			
Isolate	Designated name and GenBank accession number	Class	Amp	Kan	Strep	Generation time* (min)
JL1	*Elizabethkingia meningoseptica* JN201943	*Flavobacteria*	50	100	200	56
JL2	*Pantoea stewartii* ssp. *stewartii* EF189920	*Gammaproteobacteria*	100	< 6.25	12.5	44
JL3	*Sphingomonas* sp. JN201944	*Alphaproteobacteria*	100	12.5	> 400	150
JL4	Burkholderiaceae sp. JN201945	*Betaproteobacteria*	50	100	> 400	112
JL6	*Bacillus* sp. JN201946	*Bacilli*	100	6.25	6.25	44
Ki	*Serratia marcescens* JN201947	*Gammaproteobacteria*	100	50	> 400	27
Kiii	*Klebsiella pneumoniae* JN201948	*Gammaproteobacteria*	> 1000	12.5	12.5	30
Kiv	*Enterococcus faecalis* JN201949	*Bacilli*	ND	ND	ND	69

Amp, ampicillin; Kan, kanamycin; Strep, streptomycin; ND, not determined.
*160 r.p.m., 30 °C.

as *Elizabethkingia anophelis* (Kämpfer *et al.*, 2011), and to *Elizabethkingia meningoseptica* found in *Culex quinquefasciatus* (GenBank accession no. HQ154560). JL2 belongs to the group of *P. stewartii*- and *Pantoea agglomerans*-like bacteria commonly found in several mosquito species [(Demaio *et al.*, 1996; Pumpuni *et al.*, 1996; Straif *et al.*, 1998; Pidiyar *et al.*, 2004; Lindh *et al.*, 2005; Riehle *et al.*, 2007; Terenius *et al.*, 2008; Gusmão *et al.*, 2011); the phylogenetic relationship of the JL2 isolate to other bacteria has been shown in Terenius *et al.* (2008)]. JL6 is a close relative to *Bacillus simplex* found in *Anopheles arabiensis* (Lindh *et al.*, 2005). Ki and Kiii are close relatives to bacteria found in *Cx. quinquefasciatus*: *Serratia marcescens* (GenBank accession no. HQ154570) and *Klebsiella pneumoniae* (GenBank accession nos. HQ154554 and HQ154568), respectively.

The *ex vivo* interspecies growth inhibition showed that in general, bacterial species that had a shorter generation time could grow in plates seeded with bacteria with longer generation times (Table 3). However, JL1 had the ability to grow on plates where the seeded bacteria had a much shorter generation time. Also, JL2 and JL6 could grow on plates together with the faster-growing Ki and Kiii. In a few instances, the added bacteria also created inhibition zones. No correlation between antibiotic resistance and *ex vivo* interspecies competition was found.

Table 3. *Ex vivo* inhibition between isolates

Bacteria on plate	JL1	JL2	JL3	JL4	JL6	Ki	Kiii
Bacteria added, generation time (min)							
JL1, 56		+	+	+	+	+	+
JL2, 44	+		+	+	+	+	+
JL3, 150	−	−		W	−	−	−
JL4, 112	−	−	+		−	−	−
JL6, 44	+	+	+	+		+	+
Ki, 27	+	5.6	7.0	+	ND		+
Kiii, 30	+	+	7.9	+	+	+	

W, weak growth; ND, not determined.

The numbers indicate the radius (in mm) of an inhibition zone area created by the added bacteria.

+ = The added bacteria can grow together with the original bacteria.

− = The added bacteria cannot grow together with the original bacteria.

Dynamics of gut bacteria in mosquito cohorts

The frequent observations of *P. stewartii* and closely related species in mosquito midgut bacterial fauna (see above) suggest that *P. stewartii* type bacteria are better adapted to the mosquito gut than other bacteria. To test for coadaptation between the mosquito host and bacteria, we investigated the dynamics of four different bacterial species experimentally fed to *Ae. aegypti*, two species presumed mosquito-adapted (PsAa/PsAg and EaAg) and two nonadapted (EcHs and KoBo; see methods for details on the names and origins of bacterial species). To test for the effects of bacterial species and host sex on bacterial growth over time, we used two different modeling approaches. First, because of a large number of samples lacking bacteria, we fitted a generalized linear model to investigate the probability of bacterial growth in mosquitoes fed with bacteria (Table 4). The bacterial species main effect shows that on average, the nonadapted species (EcHs and KoBo) and the adapted species isolated from a different mosquito species (PsAg) had either similar or lower probability of being detected than the locally adapted species (PsAa). There was a marginally higher probability for detecting bacteria in male than in female hosts as indicated by the significant sex effect. While there was no overall change in bacterial presence over time, the significant two-way interaction between day and species was driven by a decrease in the probability of detecting EaAg and KoBo over time. Finally, significant three-way interactions were found between host sex, day, and two of the nonadapted species EaAg and EcHs. This was

Table 4. Parameter estimates from a generalized linear model of the presence/absence of gut bacteria as a function of time, bacterial species, and host sex. The baseline level (intercept) is the probability of PsAa bacteria being present in female hosts. See main text for further details on interpretation

	Estimates ± SD	*z*-value	*P*-value
Intercept	0.87 ± 0.17	5.15	2.64×10^{-07}
$Species_{PsAg}$	−0.94 ± 0.48	−1.94	0.0526
$Species_{EaAg}$	0.67 ± 0.66	1.02	0.3091
$Species_{EcHs}$	−0.48 ± 0.48	−0.10	0.3197
$Species_{KoBo}$	−1.30 ± 0.45	−2.86	0.0043
Sex_M	0.65 ± 0.34	1.92	0.0545
Day	−0.05 ± 0.03	−1.44	0.1489
$Species_{PsAg} \times Sex_M$	−0.87 ± 0.97	−0.90	0.3702
$Species_{EaAg} \times Sex_M$	1.25 ± 1.31	0.95	0.3428
$Species_{EcHs} \times Sex_M$	1.38 ± 0.96	1.43	0.1525
$Species_{KoBo} \times Sex_M$	0.40 ± 0.91	0.45	0.6567
$Species_{PsAg} \times$ Day	−0.16 ± 0.12	−1.35	0.1766
$Species_{EaAg} \times$ Day	−0.35 ± 0.11	−3.13	0.0018
$Species_{EcHs} \times$ Day	−0.03 ± 0.10	−0.28	0.7773
$Species_{KoBo} \times$ Day	−0.28 ± 0.10	−2.94	0.0033
$Sex_M \times$ Day	0.01 ± 0.07	0.19	0.8492
$Species_{PsAg} \times Sex_M \times$ Day	0.19 ± 0.23	0.81	0.4183
$Species_{EaAg} \times Sex_M \times$ Day	−0.42 ± 0.22	−1.87	0.0610
$Species_{EcHs} \times Sex_M \times$ Day	−0.49 ± 0.20	−2.48	0.0130
$Species_{KoBo} \times Sex_M \times$ Day	−0.21 ± 0.19	−1.12	0.2648

PsAa = *Pantoea stewartii* isolated from *Aedes aegypti*, PsAg = *P. stewartii* isolated from *Anopheles gambiae*, EaAg = *Enterobacter agglomerans* isolated from *An. gambiae*, EcHs = *Escherichia coli* isolated from *Homo sapiens*, KoBo = *Klebsiella oxytoca* isolated from *Bombina orientalis*.

Table 5. The number of individual mosquitoes containing GFP-labeled bacteria per the total number of mosquitoes sampled

		Bacterial species				
	Day*	PsAa	PsAg†	EaAg	Ec	KoBo
Females	0	6/10	8/10	6/7	2/10	5/10
	2	5/5	5/5	3/4	0/5	3/5
	6	4/5	3/5	4/5	5/5	4/5
	9	5/5	2/5	4/5	5/5	1/5
	15	3/5	–	2/5	3/5	0/5
Males	0	3/9	1/10	8/8	8/10	5/9
	2	5/5	5/5	4/5	4/5	4/5
	6	5/5	2/5	5/5	5/5	5/5
	9	5/5	3/5	5/5	5/5	2/5
	15	4/5	–	1/5‡	2/4	0/5

PsAa = *Pantoea stewartii* isolated from *Aedes aegypti* (JL2), PsAg = *P. stewartii* isolated from *Anopheles gambiae*, EaAg = *Enterobacter agglomerans* isolated from *An. gambiae*, Ec = *Escherichia coli* isolated from human gut, KoBo = *Klebsiella oxytoca* isolated from *Bombina orientalis*.

*Days after infection.

†No mosquitoes were alive on day 15.

‡Sampled on day 14.

mainly driven by the absence of these two species in early female samples (Table 5). Second, to analyze the factors determining changes in number of bacteria over time, we fitted a general linear model to the bacterial count data (Table 6). In addition to the fitted terms from the previous model (bacterial species, host sex, and day after infection), we included a second-order polynomial of day (Day^2) to reflect the initial increase and subsequent decrease in bacterial number over time as observed in all bacterial species and both host sexes. Table 6a shows overall effect of bacterial species, a nonlinear effect of time, as well as a number of significant two-way interaction effects. Details of the analyses are given in Table 6b and Fig. 1. The dynamics of bacterial number over time differed between bacterial species as indicated by the significant interaction terms between bacterial species and the linear and nonlinear effect of day (Table 6). Figure 1 shows that when comparing the two *P. stewartii* strains, the PsAa strain (isolated from the host) persisted longer in the host and reached higher numbers in the females as compared to the PsAg strain. In addition, it shows that despite belonging to the same group of bacteria and isolated from the same host species, PsAg and EaAg differed in their dynamics. Finally, Fig. 1 also shows that the dynamics of the *P. stewartii* isolates are similar and differ from the dynamics of the three other species. While the two *P. stewartii* strains grow more rapidly and to higher numbers than the other species, their number also declines relatively quickly and shows generally less variation at each time step as compared to the other species.

Table 6. ANOVA results (a) and parameter estimates and their standard errors (b) from a general linear model of temporal dynamics of gut bacteria as a function of bacterial species and host sex. The baseline level (intercept) is the mean number of PsAg bacteria counted in female hosts. See main text for further details on interpretation

(a)	d.f.	MS	*F*-value	*P*-value
Species	4	35.51	6.41	7.98×10^{-05}
Sex	1	1.78	0.32	0.5710
Day	1	4.32	0.78	0.3787
Day^2	1	125.23	22.62	4.25×10^{-06}
Sex × Day	1	24.37	4.40	0.0374
Species × Day	4	23.78	4.30	0.0025
Species × Day^2	4	27.71	5.00	0.0008
Residuals	167	5.54		

(b)	Estimates ± SD	*z*-value	*P*-value
Intercept	7.12 ± 0.26	27.08	$< 2 \times 10^{-16}$
$Species_{PsAg}$	−1.01 ± 0.96	−1.06	0.2896
$Species_{EaAg}$	−2.47 ± 0.70	−3.53	0.0005
$Species_{EcHs}$	−2.57 ± 0.69	−3.72	0.0003
$Species_{KoBo}$	−3.79 ± 0.88	−4.31	2.79×10^{-5}
Sex_M	−0.10 ± 0.36	−0.28	0.7767
Day	0.05 ± 0.05	0.94	0.3477
Day^2	−0.07 ± 0.01	−5.00	1.44×10^{-6}
Sex_M × Day	−0.17 ± 0.08	−2.10	0.0374
$Species_{PsAg}$ × Day	−0.57 ± 0.17	−3.31	0.0011
$Species_{EaAg}$ × Day	−0.49 ± 0.14	−3.58	0.0005
$Species_{EcHs}$ × Day	−0.59 ± 0.14	−4.10	7.05×10^{-5}
$Species_{KoBo}$ × Day	−0.70 ± 0.20	−3.47	0.0008
$Species_{PsAg}$ × Day^2	−0.12 ± 0.06	−2.12	0.0355
$Species_{EaAg}$ × Day^2	0.04 ± 0.02	1.59	0.1140
$Species_{EcHs}$ × Day^2	0.06 ± 0.02	2.96	0.0035
$Species_{KoBo}$ × Day^2	0.03 ± 0.06	0.49	0.6281

PsAa = *Pantoea stewartii* isolated from *Aedes aegypti*, PsAg = *P. stewartii* isolated from *Anopheles gambiae*, EaAg = *Enterobacter agglomerans* isolated from *An. gambiae*, EcHs = *Escherichia coli* isolated from *Homo sapiens*, KoBo = *Klebsiella oxytoca* isolated from *Bombina orientalis*.

Discussion

Mosquito midgut bacteria are increasingly seen as an important factor determining vector competence. For example, the presence of bacteria in the *Ae. aegypti* midgut affects its ability to harbor dengue virus. In a study by Mourya *et al.* (2002), feeding mosquitoes with dengue virus–infected blood in combination with *Aeromonas culicicola* and *E. coli* increased the percentage of dengue virus–infected mosquitoes more than twofold at day ten after infection, whereas antibiotic-treated mosquitoes showed no difference. In contrast, Xi *et al.* (2008) found that antibiotic-treated *Ae. aegypti* mosquitoes increased the titer of dengue virus more than twofold at day seven after infection. In the case of malaria, it has become increasingly clear that midgut bacteria influence the

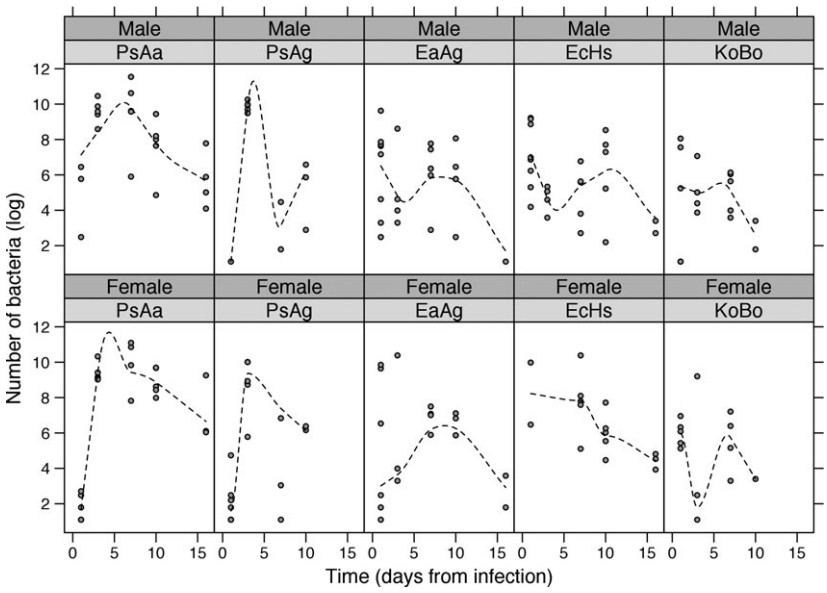

Fig. 1. Bacterial dynamics in mosquito cohorts. *Aedes aegypti* mosquitoes were fed at day 0 with GFP-labeled gut bacteria from different hosts. Each circle corresponds to the number of bacteria in one mosquito. The curve is fitted by locally weighted scatterplot smoothing (LOESS). PsAa = *Pantoea stewartii* isolated from *Aedes aegypti*, PsAg = *Pantoea stewartii* isolated from *Anopheles gambiae*, EaAg = *Pantoea agglomerans* isolated from *An. gambiae*, EcHs = *Escherichia coli* isolated from *Homo sapiens*, KoBo = *Klebsiella oxytoca* isolated from *Bombina orientalis*.

vector capacity of *An. gambiae* (Meister *et al.*, 2005; Cirimotich *et al.*, 2011; Rodrigues *et al.*, 2011). This also holds true for *An. gambiae* and bird malaria (Kambris *et al.*, 2010).

In this article, we have explored the interspecies gut flora dynamics in a laboratory colony of *Ae. aegypti* and how it is affected by competition and adaptation to the host. We isolated bacteria corresponding to six genera; the rather low number of culturable species in the mosquitoes could reflect that the environment in the laboratory is likely to be less diverse than a natural setting. Indeed, earlier laboratory studies have also retrieved a low number of bacteria with one, four, two, and six genera in Micks & Ferguson (1963), Wistreich & Chao (1963), and Gusmão *et al.* (2007, 2011), respectively. However, the sole investigation of gut flora in wild-caught adult *Ae. aegypti* has also shown that few genera were discovered either by culturing or by PCR-based methods (Zouache *et al.*, 2011). The fact that not more than three culturable species were found in a particular mosquito indicates that the internal competition could be an important factor deciding bacterial presence. One of the bacteria isolated was *S. marcescens*, which is pathogenic to several insects including *Drosophila melanogaster* (Flyg *et al.*, 1980). In bacteria-free locusts, *S. marcescens* caused disease and death, but in individuals with normal gut flora, the *S. marcescens* population steadily declined (Dillon & Charnley, 2002). Also in locusts, it was shown that a reduction in *S. marcescens* increased the species diversity of the microbiota (Dillon & Dillon, 2004). Notably, the *S. marcescens* isolate in our study (Ki) forms inhibition zones on two other species (Table 2) and could, when present, be partly responsible for a low number of species present in the midguts.

When investigating dynamics after blood feeding, the number of bacteria in each mosquito varied, and a large part of the mosquitoes did not harbor bacteria at all (Table 1). A substantial proportion of bacteria-free *Ae. aegypti* mosquitoes were also found in studies by Micks *et al.* (1961), who found bacteria in one of eleven females, and Wistreich & Chao (1963), who found bacteria in five of 21 females. In a recent study by Wang *et al.* (2011), it was shown that the composition of the gut flora in *An. gambiae* drastically changed upon a blood meal probably due to the oxidative stress that is associated with the catabolism of the blood meal. If the different *Ae. aegypti* individuals from the onset have a different gut flora composition, only those harboring bacteria that survive the oxidative stress will experience an increase in bacteria. Another possibility is that the lysozyme-like bacteriolytic factor present in the salivary glands and in the crop of male and female *Ae. aegypti* (Rossignol & Lueders, 1986) is expressed at different levels in individual mosquitoes leading to a

hostile environment for the gut bacteria in certain individuals. Likewise, in *An. gambiae* and *D. melanogaster*, the down-regulation of the immunoglobulin (Ig)-superfamily receptor Down syndrome cell adhesion molecule AgDscam and Dscam, respectively, radically increases the number of bacteria in their hosts (Watson *et al.*, 2005; Dong *et al.*, 2006). Anticipating a similar activity also in *Ae. aegypti*, individual differences in its expression would again give individual-specific prospects for the bacteria to grow.

By feeding cohorts of *Ae. aegypti* with several gut bacteria of different host origins, we could observe differences in how long and in what numbers the bacteria persisted in the cohort. Previously, St. John *et al.* (1930) investigated the survival of *Staphylococcus aureus*, *Bacillus prodigiosus*, and *Bacillus leprae* in laboratory-reared *Ae. aegypti*. The bacteria were fed mixed with blood, and all species could survive in the midgut at least 24 h. *B. prodigiosus* was exceptional and survived for 3 days. In our study, the data suggest that *P. stewartii* isolated from *Ae. aegypti* had the best ability to remain in the cohort. In other insects, the closely related species *P. agglomerans* commonly exist in a symbiotic relationship. For example, it was shown to persist for more than 50 generations (2 years) in the Western Flower Thrips, *Frankliniella occidentalis*, whereas bacteria ingested with the food did not remain to the next generation (de Vries *et al.*, 2001). Another observation is that *P. agglomerans* colonization in germ-free locusts was supported by the presence of *K. pneumoniae* (Dillon & Dillon, 2004), a bacterium found in our study, which adds to the notion that bacteria can either support or suppress the growth of other species (c.f. the potential impact of *S. marcescens* discussed above). In laboratory-reared *An. gambiae* mosquitoes, *P. stewartii* was picked up from the water by newly emerged females and was also shown to emit volatiles that conferred a positive oviposition response (Lindh *et al.*, 2008). By this, the bacteria remained in the cohort over generations. In *An. gambiae*, Riehle *et al.* (2007) could show that after four selective mosquito passages of a *P. agglomerans* isolated from laboratory-reared *Anopheles stephensi*, the survival increased from 2 to 14 days, again pointing to the fact that *P. agglomerans* bacteria can easily adapt to their host. In our study, we show that this adaptation is specific and closely linked to the host origin. The ability of bacteria isolated from one mosquito host to survive in another was previously investigated by Crotti *et al.* (2009). They showed that *Asaia* isolated from *Ae. aegypti* could colonize 100% of the *An. stephensi* individuals tested. However, the reverse was not true with only 58% of the *Ae. aegypti* infected with *Asaia* from *An. stephensi*, and surprisingly, the same strain of *An. stephensi Asaia* was more efficient in its colonization of the evolutionary distant hemipteran *Scaphoideus titanus* (74% of the individuals; Crotti *et al.*, 2009), whereas our data indicate that the shorter evolutionary distance between *Ae. aegypti* and the original host of the bacterial isolate, the better the survival.

There are a number of factors that could affect bacterial growth and interspecies competition in the mosquito midgut. Some examples are sugar source utilization, the extent of growth after a blood meal, and the level of resistance to inhibiting factors produced by other bacteria or by the mosquito. To further understand the interspecies competition between bacteria in the mosquito, it would be interesting to use bacteria expressing different markers and analyze the midgut content both *ex vivo* and *in vivo*. In conclusion, the results presented here highlight the ability of midgut bacteria to adapt to their host. As a consequence, internal competition may affect the microbial community both in the individual mosquito and in the entire mosquito population.

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