The mosquito adulticidal *Chromobacterium* sp. Panama causes transgenerational impacts on fitness parameters and elicits xenobiotic gene responses

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**Abstract**

Background: Vector control is critical in reducing the disease burden caused by mosquitoes, and insecticides are an effective tool to control vector populations. Resistance to common insecticides is now widespread, and novel classes of insecticides are needed. In previous work, we described the mosquitocidal activity of *Chromobacterium* sp. Panama (*C*. *sp_P), a bacterium found in association with mosquitoes in natural populations. In the current work, we further explored the effects of exposure to the bacterium on mosquito fitness and mosquito physiology.

Results: We found that *C*. *sp_P* has mosquitocidal activity against a broad range of mosquito taxa. When exposed to *C*. *sp_P* as adults, female *An. gambiae* suffered reduced longevity, but experienced no change in fecundity. The offspring of these females, however, had higher mortality as larvae and were slower to develop compared to offspring of control females. We also found that the mosquitocidal activity of *C*. *sp_P* was retained after removal of live cells from biofilm culture media, suggesting the bacteria secrete mosquitocidal compound(s) into the media during growth. Exposure to this cell-free *C*. *sp_P*-conditioned media caused female midgut transcriptional changes comprising detoxification, xenobiotic response, and stress response genes, suggesting the physiological response to *C*. *sp_P* is similar to that of insecticide exposure. Finally, we found that multiple members of the *Chromobacterium* genus had mosquitocidal activity, but this activity was highest in mosquitoes treated with *C*. *sp_P*.

Conclusions: Our findings suggest that *C*. *sp_P* produces factor(s) with strong effects on mosquito longevity and fitness, which may be of interest for mosquitocide development. More generally, they indicate that further exploration of mosquito-associated and environmental microbes for novel insecticidal compounds or biocontrol agents is warranted.

Keywords: *Anopheles gambiae*, *Chromobacterium*, Mosquito, Host-microbe interactions, Mosquitocide, Vector control, Transcriptome
and, in addition to other environmental bacteria and fungi, may constitute a potential source of novel biocontrol and/or mosquitocidal agents.

In previous work, we described *Chromobacterium* sp. Panama (*C*. sp._P_), isolated from the midguts of *Aedes aegypti* mosquitoes in Panama [17]. Bacteria from this genus are soil-dwelling, Gram-negative microbes, and are highly recognizable by the characteristic purple pigment called violacein produced by many members of the genus (though notably not by *C*. sp._P_) [17, 18]. The most well-described member of the genus, *C. violaceum*, has been found to produce numerous bioactive factors with antimicrobial properties, as well as hydrogen cyanide, which can be used to “bio-leach” gold from discarded electronics [18, 19]. In our previous study, we found *C*. sp._P_ to have strong mosquitocidal activity against *Anopheles gambiae* (a primary vector of the malaria parasite *Plasmodium falciparum*) and *Aedes aegypti* (the primary vector of dengue and Zika viruses) adult females when exposed to the bacteria in a sugar meal [17]. Additionally, when present in the mosquito midgut, *C*. sp._P_ reduced susceptibility of *An. gambiae* and *Ae. aegypti* mosquitoes to *Plasmodium falciparum* and dengue virus, respectively [17]. These anti-pathogen properties are active *in vitro* (i.e. independent of the mosquito), suggesting the bacteria produces compound(s) with anti-pathogen activity [17]. Another member of the genus, *C. subtsugae*, has been shown to have insecticidal activity across diverse taxa of insects, including beetles, moths and whiteflies, though it has not been shown to be active against mosquitoes [20]. Additionally, a patent has been filed reporting that *C. vaccinii* is active against moths and *Ae. aegypti* mosquito larvae [21].

In the present study, we further explored the mosquitocidal properties of *C*. sp._P_ and its effects on mosquito fitness and mosquito physiology as measured by transcriptome changes upon exposure. We found that *C*. sp._P_ has broad insecticidal activity among mosquitoes, inducing mortality in three additional species of vector mosquitoes. We also found that *An. gambiae* females that survived exposure to *C*. sp._P_ suffered reduced fitness potential; their offspring had increased mortality as larvae and showed delayed time to pupation and eclosion. We determined that mosquitocidal factor(s) of *C*. sp._P_ persisted in cell-free preparations of bacteria cultured in biofilm conditions, and found that exposure to cell-free preparations of *C*. sp._P_ increased transcript abundance in genes related to detoxification and insecticide treatment and decreased transcript abundance of genes related to nucleosome and chromatin formation. Finally, we determined that other species in the *Chromobacterium* genus have mosquitocidal properties, suggesting production of insecticidal compounds is common throughout the genus.

**Methods**

**Mosquito strains and maintenance**

*Anopheles gambiae* (Keele strain), *An. stephensi* (Liston strain), *Ae. albopictus* (Gainsville strain, MRA-804 from BEI Resources), and *Culex quinquefasciatus* (JHB strain, NR-43025 from BEI Resources) were reared at 27 °C and 80% RH with a 14:10 light:dark photocycle.

**Bacterial information**

*C*. sp._P_ was isolated from *Aedes aegypti* in Panama in 2010 [17, 22]. *Pantoea* sp. was isolated from *Anopheles arabiensis* in Zambia in 2010 [23]. *Chromobacterium violaceum* was obtained from ATCC (strain: ATCC 12472), and other species of *Chromobacterium* were obtained from the Leibniz Institute DSMZ: *C. aquaticum* (DSM 19852), *C. subtsugae* (DSM 17043) and *C. vaccinii* (DSM 25150).

**Bacterial culture growth**

Bacteria were grown either in “planktonic” or “biofilm” conditions. To culture bacteria in planktonic conditions, we added 1 μl of pure freezer stock to 5 ml of LB and incubated with shaking at 30 °C for 16–18 h or 72 h, as indicated. The only exception to this protocol was for the experiment using multiple mosquito species, in which the planktonic culture was grown by inoculating 15 ml LB broth with 150 μl fresh culture and incubating at 30 °C for ~18 h with shaking. The fresh culture was grown by inoculating 5 ml LB with several single colonies grown from glycerol freezer stock and incubating overnight at 30 °C with shaking. To culture bacteria in biofilm conditions, we added 1 μl of pure freezer stock to 5 ml LB in a sterile 6-well cell culture plate and incubated without shaking at 30 °C for 72 h.

**Bacterial culture preparation**

Planktonic cultures were pelleted and washed twice with 1× PBS then re-suspended in additional 1× PBS to 1.0 (± 0.1) OD<sub>600</sub> and if necessary diluted or concentrated to achieve a desired cell density (as indicated). For the experiment using filtered planktonic culture, the culture was diluted to 1.0 (± 0.1) OD<sub>600</sub> using sterile LB and not washed to preserve the supernatant. To collect biofilm, liquid media was removed from each well of the culture plate and 1 ml 1× PBS was added per well. Biofilm was removed from the culture plate by repeatedly pipetting the 1× PBS up and down in each well. Biofilm suspension was then transferred to a centrifuge tube and vortexed for 1 min to further encourage suspension in 1× PBS. To collect biofilm supernatant, liquid media was removed from biofilm culture wells, transferred to centrifuge tubes and vortexed for 1 min. To filter each preparation, cultures were pelleted at 5000 rpm for 3–5 min
and the liquid supernatants were passed through a 0.2 μm syringe filter. To measure CFU/ml of each bacterial culture preparation, an aliquot of unfiltered culture was serially diluted in 1× PBS and plated on LB agar. Colony forming units were counted from each dilution that yielded countable colonies and averaged to calculate CFU/ml. For C.sp_P, diluting overnight planktonic culture to 1.0 OD600 resulted in an average of 4.59 × 10^8 CFU/ml (range of CFU/ml measurements: 1.25 × 10^7 to 9.75 × 10^8). When a higher or lower cell density was needed, CFU/ml was determined post-hoc, and in those instances CFU/ml is indicated in the respective figure legend. On average, C.sp_P biofilm suspension contained 5.03 × 10^8 CFU/ml (range of CFU/ml measurements: 1.30 × 10^8 to 1.42 × 10^9), and biofilm supernatant contained 8.60 × 10^7 CFU/ml (range of CFU/ml measurements: 5.0 × 10^6 to 1.45 × 10^8).

To test whether the mosquitocidal factor contained in the C.sp_P supernatant was volatile, C.sp_P biofilm supernatant and sterile LB were filter sterilized and aliquoted into 1.5 ml microcentrifuge tubes and the open tubes were centrifuged for 30 min under a vacuum in a Vacufuge (Eppendorf, Hamburg, Germany). Noncentrifuged controls for both sample types were maintained at room temperature with lids sealed. Loss volume of vacuum centrifuged samples was reconstituted by adding sterile water to the centrifuged samples to the same final volume as samples that were not centrifuged. All four preparations were fed to An. gambiae females in sucrose meals as described below.

Measuring short-term survival in adults after bacterial exposure

Adult females were cold anesthetized 3–7 days after eclosion, sorted into cardboard cups, and provided 10% sucrose ad libitum until the experiment. Mosquitoes were starved overnight to encourage feeding, and the morning of the experiment cultures were harvested and prepared as described above. Bacterial culture preparations were added to sterile sucrose, and 1× PBS or LB media were added in place of bacteria as controls. Unless otherwise stated, bacterial preparations (unfiltered and filtered), PBS and LB were mixed 1:1 with 3% sucrose, and in all cases the final sucrose concentration was 1.5%. All sugar meals were provided to females for 24 h in microcentrifuge tubes containing sterile filter paper wicks. Experimental sugar meals were then removed, and all treatments were provided 10% sucrose ad libitum. Survival was monitored for seven to ten days after treatment commenced.

Blood-feeding

Blood meals were prepared by mixing 40% human red blood cells and 60% heat inactivated human serum. Mosquitoes were starved 6–8 h before being allowed to blood-feed for 1 h using membrane feeders as described previously [24]. After feeding, females were cold anesthetized and unfed females were removed from the experiment.

Fecundity and oviposition experiments

Six-to seven-day old females were fed a 10^7 dose of C. sp_P or P. sp, or PBS as a control, for 24 h and each treatment was blood-fed approximately 56 h post-exposure. This dose of C.sp_P was chosen because it is high enough to ensure efficient exposure and moderate mortality in An. gambiae, but low enough that sufficient numbers of females survive through completion of the experiment. Females that did not blood-feed were removed from the experiment. Three days later, blood-fed females were placed into individual oviposition cups (50 ml conical bottom tubes containing 5–7.5 ml of deionized water and a piece of filter paper). After being allowed to oviposit for two days, the number of ovipositing females and the number of eggs oviposited were recorded. If females oviposited ten or fewer eggs, insemination was verified by spermatheca dissection. The spermathecae were dissected in 30 μl of 1× PBS then transferred to 10 μl of Geimsa stain (diluted 1:100 in deionized water) and physically crushed onto a glass slide using ethanol-sterilized forceps, allowed to air dry, fixed with 50 μl of methanol for 30 min, and rinsed with deionized water. Slides were viewed using a light microscope at 10–40×. Only inseminated females were included in the oviposition and fecundity analyses, and rate of insemination did not significantly differ between the treatment groups (χ^2 = 4.77, df = 2, P = 0.092, Additional file 1: Figure S1).

Development and life history experiments

Adult An. gambiae females were fed a 1.5% sucrose solution containing C.sp_P at a cell density of 10^7 CFU/ml or 1× PBS as a control for 24 h. Approximately two days after C.sp_P exposure, females were then given a blood meal and allowed to oviposit. Eggs were hatched and 100 larvae per treatment were transferred to clean trays. Larval survival and pupation events were recorded. Three 1 ml samples of water were collected from the oviposition water, larval trays before adding the food and larvae, and larval trays at 4 and 8 days after transferring larvae to the trays. Water samples were plated onto LB agar plates at 10-fold serial dilutions to calculate CFU, and unique colonies were isolated and identified to bacterial species using the 16S rDNA sequence [25]. Pupae were then transferred to a small beaker in a cage and pupal survival and eclosion events were monitored. Upon eclosion, male and female adults were transferred
to separate cups (18–23 individuals per cup) and survival was monitored until all adults were dead.

**Microarray experimental design, sample preparation and analysis**

Six to seven day old adult *An. gambiae* females were starved overnight and provided sugar meals containing 3% sucrose mixed 1:1 with either LB or 72 h *C. sp_P* cell-free biofilm supernatant (final sucrose concentration 1.5%). Both LB and *C. sp_P* supernatant were filter sterilized before being added to the sucrose solution. After 24 h, midguts were dissected from 20 adult females per treatment in sterile 1× PBS on a cold block and pooled in TRIzol (Invitrogen, Carlsbad, USA) reagent on ice. Midguts were stored at -80 °C until RNA extraction. The experiment was repeated four independent times, with one pool of 20 midguts collected per treatment per replicate. RNA was extracted following the manufacturer’s protocol and genomic DNA was removed using the TURBO DNA-free Kit (Invitrogen) according to the manufacturer’s instructions. Quality of RNA was verified using an Agilent Bioanalyzer 2100. Transcriptome analysis was performed using a custom Agilent microarray described previously [26, 27]. All samples were labeled using the Two-Color Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, USA) according to the manufacturer’s instructions. 200 ng of RNA from each sample was used as input for the labeling reaction and labeled cRNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Hybridization was performed according to Agilent’s Two-Color Microarray-Based Gene Expression Analysis Protocol. RNA extracted from LB-fed individuals was hybridized to that of individuals fed cell-free *C. sp_P* biofilm supernatant collected in the same replicate experiment, and samples were labeled in a dye-swap design to prevent dye bias. Feature extraction was performed using an Agilent Scanner and Agilent Feature Extraction Software. Analysis of microarray data was performed as in [28]. In brief, differential transcript abundance between LB-fed and *C. sp_P*-fed female midguts was assessed using limma in R [29] after background correction using the “normexp” method [30] and after normalizing signals using global loess within array normalization [31]. Lists of genes with differential transcript abundance between treatments were then assessed for Gene Ontology term enrichment using DAVID [32, 33].

**Statistical analysis**

Unless otherwise stated, Cox proportional hazards models were used to assess the effect of treatment on survival. Individuals that were excluded during the experiment or were still alive at the conclusion of the experiment were treated as censored data. For all analyses, experimental replicate was included as a co-factor to account for variation across replicate experiments. For the experiments testing the effect of multiple species of *Chromobacterium* on survival and the effect of *Chromobacterium* sp. Panama and *Pantoea* sp. on survival, the data did not meet the assumptions of a proportional hazards model and therefore pairwise Log-Rank tests followed by a multiple testing correction were used. For the experiments testing survival of offspring and pupation/eclosion rates, Log-Rank tests were performed. All Cox proportional hazards and Log-Rank tests were performed in R [34]. Fecundity and oviposition data were analyzed by Kruskal-Wallis test in R and one-way ANOVA in GraphPad Prism. Larval bacterial load data and bacterial load data from biofilm versus supernatant were analyzed by two-way ANOVA in R.

**Results**

We have shown in previous work that ingestion of *Chromobacterium* sp. Panama (*C. sp_P*) causes reduced longevity in adult *An. gambiae* and *Ae. aegypti* mosquitoes [17]. We were interested in further exploring the effects of *C. sp_P* on mosquito fitness and its adulticidal activity. For this, we investigated how exposure to *C. sp_P* during adulthood influences fecundity of *An. gambiae* females as well as development rate and survival of their offspring. We then probed the nature of the adulticidal activity of *C. sp_P*, and the effects of compounds secreted by *C. sp_P* on the transcriptome of adult female *An. gambiae*. We also explored other members of the genus *Chromobacterium* to determine whether species related to *C. sp_P* display adulticidal activity as well.

**C. sp_P** has adulticidal effects against a broad range of disease vector mosquitoes

In previous work, we showed that oral exposure to *C. sp_P* caused reduced longevity in *An. gambiae* and *Ae. aegypti* mosquitoes. We investigated the effects of oral exposure to *C. sp_P* on three additional species of disease vector mosquitoes: *Ae. albopictus*, *Culex quinquefasciatus* and *An. stephensi*. We allowed adult females to feed on two different densities of *C. sp_P*, or PBS as a control, in a sugar meal for 24 h and monitored survival for ten days post-exposure. We found that *An. stephensi* showed significantly reduced survival after exposure to *C. sp_P* at both a lower (10^5 CFU/ml) and a higher (10^10 CFU/ml) bacterial cell density, while the survival of *Ae. albopictus* and *C. quinquefasciatus* was significantly reduced only after exposure to *C. sp_P* at a density of 10^10 CFU/ml (Fig. 1).

**C. sp_P** exposure has no effect on fecundity among surviving females but reduces larval survival and slows development of their offspring

*C. sp_P* exposure causes significant mortality among adult *An. gambiae* females, but we were interested in
determining whether females that survive suffer residual effects on fitness traits. We exposed adult *An. gambiaceae* females to *Csp*-*P* in a 1.5% sugar meal at a density of $10^7$ CFU/ml, which causes moderate mortality (Fig. 2a). Simultaneously, we exposed adult *An. gambiaceae* to *Pantoea* sp. bacteria which does not cause mortality at the same cell density (Fig. 2a), to control for the potential nutritional impact of bacterial ingestion on egg production. We then blood-fed females from all treatments and assessed probability of oviposition and average total number of eggs laid per female. We found that *Csp*-*P* exposure had no effect on probability of oviposition or number of eggs laid relative to either the PBS- or *Pantoea* sp.-exposed controls (Fig. 2b, c).

In a subsequent experiment, we exposed adult female mosquitoes to *Csp*-*P* in a 1.5% sugar meal at a density of $10^7$ CFU/ml and then allowed them to blood-feed and lay eggs. We recorded the rate of development and monitored survival of the offspring across all stages of development. We found that the proportion of eggs that hatched was not significantly different between treatments; mean hatch rate for PBS was 0.612 (95% CI: 0.75–0.48), while for *Csp*-*P* it was 0.628 (95% CI: 0.73–0.53, t = 0.902, df = 4, P = 0.418). However, larval mortality was significantly higher for offspring of *Csp*-*P*-exposed mothers compared to offspring of PBS-exposed mothers (Fig. 3a, $\chi^2 = 43.1, df = 1, P < 0.0001$). Additionally, the rate of pupation was significantly slower for offspring of *Csp*-*P*-exposed mothers relative to that of PBS-exposed mothers; the median time to pupation was nine days and eight days, respectively (Fig. 3a, $\chi^2 = 101, df = 1, P < 0.0001$). Survival was not significantly different between the groups at the pupal stage, however, time to eclosion was significantly longer in offspring of *Csp*-*P*-exposed mothers (median time to eclosion = 11 days) compared to offspring of PBS-exposed mothers (median time to eclosion = 9 days) (Fig. 3b, $\chi^2 = 103, df = 1, P < 0.0001$). Survival of F1 adults was similar between the two groups (Fig. 3c). We sampled oviposition water and found live *Csp*-*P* in 0 of 9 samples collected over three replicates. We also sampled larval water at days 0, 4, and 8 post-larval transfer and found live *Csp*-*P* in one sample taken from a single replicate on day four. All other larval water samples were negative for *Csp*-*P*. In addition to testing for *Csp*-*P* in these water samples, we also quantified CFU/ml of all bacteria that grew on LB agar from each water sample and found that overall bacterial load did not significantly differ between treatments (Additional file 2: Figure S2).

**Csp*-*P* mosquitocidal activity persists after removal of live bacteria when grown in biofilm but not planktonic conditions**

We were interested in further exploring the nature of the mosquitocidal compound(s) produced by *Csp*-*P* to better understand the mechanism by which it causes mosquito mortality. Because treatment with live bacteria introduces substantial variability and potential for dynamic rates of exposure over time, we tested whether *Csp*-*P* secretes the mosquitocidal activity(ies) into the culture media by assaying whether cell-free preparations exerted mosquitocidal activity. We grew *Csp*-*P* in planktonic culture for approximately 16 h or 72 h and filtered live bacteria from an unwashed aliquot of each culture. We then provided *An. gambiaceae* females with 1.5% sugar meals containing filtered and unfiltered preparations from each incubation time or LB as a control.

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**Fig. 1** Csp*-*P* exposure causes mortality across a broad taxonomic range of disease vector mosquitoes. Planktonic overnight cultures of Csp*-*P* were washed two times with 1× PBS and diluted or concentrated to obtain “low” (i.e. approximately $10^3$ or $10^4$ CFU/ml) and “high” (i.e. approximately $10^9$ CFU/ml) bacterial cell densities, as per our previous work [17]. Bacteria of each density or 1× PBS as a control were then mixed 1:1 with 3% sucrose (final sucrose concentration 1.5%) and provided to adult females for 24 h, at which point all treatments were given approximately $10^{10}$ CFU/ml. Bacteria of each density or 1× PBS was washed two times with 1× PBS and diluted or concentrated to obtain "low" (i.e. approximately $10^6$ CFU/ml) bacterial cell densities, as per our previous work [17]. Bacteria of each density or 1× PBS were then added to 1× PBS and diluted or concentrated to obtain "low" (i.e. approximately $10^3$ or $10^4$ CFU/ml) bacterial cell densities. Bacteria of each density or 1× PBS were then added to 1% sucrose (final sucrose concentration 1.5%) and provided to adult females for 24 h, at which point all treatments were given 1% sucrose. *Aedes albopictus* females were exposed to Csp*-*P* 10$^6$, PBS vs Csp*-*P* 10$^6$, z = 1.93, P = 0.0531; PBS vs Csp*-*P* 10$^{10}$, z = 6.67, P < 0.0001. *Culex quinquefasciatus* females were exposed to Csp*-*P* 10$^6$, PBS vs Csp*-*P* 10$^6$, z = -1.06, P = 0.289; PBS vs Csp*-*P* 10$^{10}$, z = 9.30, P < 0.0001. *Anopheles stephensi* females were exposed to Csp*-*P* 10$^6$, PBS vs Csp*-*P* 10$^6$, z = 2.87, P = 0.004; PBS vs Csp*-*P* 10$^{10}$, z = 10.26, P < 0.0001. For each experiment, treatments and controls were repeated 3 times with 25–30 individuals per replicate, while was repeated 5 times with 9–20 individuals per replicate. Survival curves were fitted using the Kaplan-Meier method with pooled data from all replicates. Vertical tick-marks indicate censored samples. Data from all experiments were analyzed using a Cox proportional hazards model including treatment and replicate factors.
We found that both unfiltered cultures caused significant mortality over seven days compared to LB (Fig. 4a; C. sp_P\textsubscript{16hr Unfilt}, \(z = 7.11, P < 0.0001\); C. sp_P\textsubscript{72hr Unfilt}, \(z = 9.57, P < 0.0001\)), but that when live bacteria were filtered out of the culture, survival was either no different or significantly better than the control (Fig. 4a; C. sp_P\textsubscript{16hr Filt}, \(z = -1.76, P = 0.078\); C. sp_P\textsubscript{72hr Filt}, \(z = -3.25, P = 0.001\)).

We also grew C. sp_P in biofilm conditions (30 °C, without shaking) for 72 h and investigated whether adulticidal activity is present under these growth conditions and whether it persists after removal of live bacterial cells. We found that An. gambiae females given C. sp_P biofilm re-suspended in a sugar meal had significantly reduced survival relative to the PBS control (Fig. 4b, \(P = 2.0 \times 10^{-16}\)). We also filtered the biofilm resuspension as well as the biofilm supernatant (i.e. the LB media in which the biofilm grew) to remove live cells and exposed An. gambiae females to sugar meals containing each cell-free preparation. We found that filtering eliminated adulticidal activity from C. sp_P biofilm, but not from C. sp_P biofilm supernatant when compared to LB (Fig. 4b, PBS vs C. sp_P\textsubscript{Biofilm Filt}, \(z = 0.83, P = 0.18\), LB vs C. sp_P\textsubscript{Biofilm Sup. Filt}, \(z = 6.66, P < 0.0001\)).

C. sp_P–produced hydrogen cyanide does not mediate the adulticidal activity

Hydrogen cyanide (HCN) is a known secondary metabolite of multiple Chromobacterium species [21, 35]. HCN is a generalist poison and is therefore one potential source of mosquitocidal activity of C. sp_P against adults. We tested cell-free C. sp_P biofilm supernatant for hydrogen cyanide and found the average concentration to be 0.039 mg/l (range: 0.023–0.047 mg/l). The
The concentration of HCN in the sugar meals fed to adults in our experiments is therefore approximately 0.02 μg HCN/ml. Hydrogen cyanide is a gas and boils at 25.6 °C. We therefore hypothesized that if HCN is causing adult mosquito mortality, vacuum centrifugation would eliminate the mosquitocidal effect due to evaporation of HCN. We centrifuged *C. sp*. P filtered biofilm under a vacuum in open microcentrifuge tubes to allow evaporation and tested the impact of vacuum centrifugation on mosquitocidal activity. We found that exposure to *C. sp*. P filtered biofilm supernatant reduced survival regardless of whether the sample had been vacuum centrifuged (Fig. 5, *C. sp*. P vs LB, $z = 5.95$, $P < 0.0001$; *C. sp*. P vs LB, $z = 7.11$, $P < 0.0001$). Vacuum centrifugation also reduced survival, and this was consistent across the experimental and control treatments (Fig. 5, $z = 4.08$, $P < 0.0001$).

**Exposure to C. sp. P filtered biofilm supernatant alters transcript abundance in genes important for detoxification, insecticide resistance, and stress response**

To gain insight on how *C. sp*. P affects adult females, we performed a genome-wide transcriptome analysis comparing transcript abundance of females fed a sugar meal.
containing filtered C.sp_P (i.e. cell free) biofilm supernatant versus LB as a control. Transcript abundance was analyzed in midgut tissues harvested 24 h after introduction of experimental sugar meals. We found that exposure to cell-free C.sp_P biofilm supernatant caused altered transcript abundance of 62 genes involved in stress response (R/S/M), 79 involved in metabolism (MET) and 59 involved in replication, transcription, and translation (R/T/T) (Fig. 6, Additional file 3). A gene ontology enrichment analysis confirmed these observations. Genes significantly up- or downregulated by cell-free C.sp_P biofilm supernatant exposure were enriched for Biological Process GO terms related to “response to xenobiotic stimulus” and “response to insecticide” (Table 1). This was primarily driven by the upregulation of multiple cytochrome P450 genes; 14 members of the CYP6 subfamily were significantly upregulated in response to C.sp_P treatment, as were members of the CYP4 and CYP9 subfamilies (Additional file 3). Other Biological Process GO terms that were significantly enriched include those related to “organic acid metabolic process.” Cellular Component GO terms significantly overrepresented included those related to “chromatin,” “DNA packaging complex,” and “protein DNA complex,” a result driven by the downregulation of multiple genes coding for histone proteins and DNA repair proteins (Table 1, Additional file 3).

Oral exposure to multiple Chromobacterium species causes mortality in adult mosquitoes

At least two species of Chromobacterium, C. subtsugae and C. vaccinii, have been shown to have insecticidal properties, and C. subtsugae is currently being used as a biocontrol agent marketed as Grandevo® (Marrone BioInnovations) [20, 21, 36]. Chromobacterium vaccinii causes mortality of Aedes aegypti larvae when added to the larval breeding water [21]. Chromobacterium subtsugae was shown to cause mortality in diverse insect taxa, though not in the larvae of Culex pipiens, the only mosquito species on which it was tested [20]. We investigated whether other species of Chromobacterium in addition to C.sp_P induce mortality in An. gambiae adults and whether the mosquitoicidal activity is maintained after removal of live cells, as it is in C.sp_P. To test this, we grew five bacterial species in biofilm conditions for 72 h: C.sp_P, C. aquaticum, C. subtsugae, C. violaceum and C. vaccinii. We then isolated the biofilm as well as the surrounding media (biofilm supernatant) from each species and provided filtered (i.e. cell-free) and unfiltered preparations to adult An. gambiae females in sugar meals for 24 h. CFUs per ml were similar among the species for each fraction, but across all species, the biofilm fraction harbored more CFUs than the supernatant ($F_{(1, 23)} = 11.26, df = 1, P = 0.0029$, Additional file 4: Figure S3). We found
that unfiltered biofilm and biofilm supernatant of all species caused significant mortality over seven days, with most of the mortality induced in the first 72 h (Fig. 7). Biofilm supernatant from C. sp_P had the strongest mosquitocidal activity, causing 100% mortality by 48 h (Fig. 7a). Significant mosquitocidal activity was retained in the cell-free filtrates of C. sp_P (Fig. 7a). Significant mosquitocidal activity was retained in the cell-free filtrates of C. sp_P biofilm supernatant (Fig. 7a), C. subtsugae biofilm supernatant (Fig. 7c), and C. vaccinii biofilm (Fig. 7e). Removing live cells by filtration eliminated all mosquitocidal activity from C. aquaticum (Fig. 7b) and C. violaceum (Fig. 7d).

**Discussion**

Exposure to C. sp_P causes significant mortality in the disease vector mosquitoes An. gambiae and Aedes aegypti [17]. In this study, we aimed to further elucidate the effects of the bacteria on vector mosquitoes. Our first goal was to assess whether exposing females to a sub-lethal dose of C. sp_P had any effects on their fecundity, fertility, or survival among their F1 offspring. We therefore blood-fed C. sp_P- or PBS-exposed females, collected their eggs, and reared F1 offspring to evaluate transgenerational fitness effects. We found that exposure of adult female An. gambiae to C. sp_P had no effect on probability of oviposition, average number of eggs laid, or the percentage of eggs that hatched. However, maternal exposure to C. sp_P was associated with increased larval mortality, delayed pupation, and delayed eclosion of F1 offspring. Maternal C. sp_P exposure had no effect on the longevity of F1 adult offspring. These results suggest that sub-lethal C. sp_P exposure of adult females causes transgenerational effects on their offspring during immature stages of development.

Bacteria present in adult females have been found on the surface of mosquito eggs [9], and it is therefore possible that C. sp_P may have been vertically transmitted to larval offspring by egg smearing or that C. sp_P could have been present in the larval breeding water...
throughout development. *C*. sp *P* was not detected in oviposition water and was only detected in larval water in a single measurement from one of three replicates, suggesting that *C*. sp *P* was generally not present in oviposition or breeding water of F1 larvae. It is possible that *C*. sp *P* was present transiently or in low levels that we failed to detect, and we therefore cannot exclude that *C*. sp *P* directly influenced larval mortality and developmental delays via vertical transmission or environmental exposure. However, the transgenerational fitness effects were consistent across replicates regardless of whether *C*. sp *P* was detected in larval water, suggesting these

| GO term ID  | GO term name                                      | Count | Fold enrichment | BH corrected P-value |
|-------------|---------------------------------------------------|-------|----------------|----------------------|
| Biological process                                     |                                    |       |                |                      |
| GO:0044712 | Single-organism catabolic process                 | 30    | 2.35           | 3.72E-03             |
| GO:0043436 | Oxoacid metabolic process                         | 39    | 2.13           | 3.85E-03             |
| GO:0042178 | Xenobiotic catabolic process                       | 10    | 6.20           | 4.30E-03             |
| GO:0071466 | Cellular response to xenobiotic stimulus          | 10    | 6.20           | 4.30E-03             |
| GO:006805  | Xenobiotic metabolic process                       | 10    | 6.20           | 4.30E-03             |
| GO:0019752 | Carboxylic acid metabolic process                 | 38    | 2.09           | 4.33E-03             |
| GO:0006082 | Organic acid metabolic process                     | 40    | 2.00           | 4.81E-03             |
| GO:0009410 | Response to xenobiotic stimulus                   | 10    | 5.94           | 5.17E-03             |
| GO:0009407 | Toxin catabolic process                            | 10    | 6.48           | 5.57E-03             |
| GO:0090487 | Secondary metabolite catabolic process             | 10    | 6.48           | 5.57E-03             |
| GO:0017085 | Response to insecticide                           | 10    | 6.48           | 5.57E-03             |
| GO:0009404 | Toxin metabolic process                            | 10    | 6.48           | 5.57E-03             |
| GO:0017143 | Insecticide metabolic process                      | 10    | 6.48           | 5.57E-03             |
| GO:0046701 | Insecticide catabolic process                      | 10    | 6.48           | 5.57E-03             |
| GO:0046680 | Response to DDT                                   | 10    | 6.48           | 5.57E-03             |
| GO:0009636 | Response to toxic substance                        | 11    | 5.81           | 9.92E-03             |
| GO:0006334 | Nucleosome assembly                                | 10    | 4.32           | 4.72E-02             |
| Molecular function                                      |                                    |       |                |                      |
| GO:0016705 | Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen | 25    | 2.45           | 2.08E-02             |
| GO:0004497 | Monoxygenase activity                              | 21    | 2.62           | 2.47E-02             |
| GO:0005506 | Iron ion binding                                  | 25    | 2.50           | 3.08E-02             |
| GO:0046906 | Tetrapyrrole binding                              | 22    | 2.34           | 4.92E-02             |
| GO:0020037 | Heme binding                                      | 22    | 2.35           | 5.60E-02             |
| Cellular component                                       |                                    |       |                |                      |
| GO:0032993 | Protein-DNA complex                               | 20    | 6.01           | 2.02E-08             |
| GO:0000786 | Nucleosome                                        | 16    | 6.45           | 4.20E-07             |
| GO:0044815 | DNA packaging complex                             | 16    | 5.65           | 2.52E-06             |
| GO:0000785 | Chromatin                                         | 20    | 3.98           | 1.35E-05             |
| GO:0000790 | Nuclear chromatin                                 | 13    | 5.40           | 8.46E-05             |
| GO:0005694 | Chromosome                                        | 29    | 2.48           | 3.21E-04             |
| GO:0000788 | Nuclear nucleosome                                | 6     | 14.12          | 3.70E-04             |
| GO:0000228 | Nuclear chromosome                                | 17    | 3.20           | 1.50E-03             |
| GO:0044427 | Chromosomal part                                  | 25    | 2.42           | 1.75E-03             |
| GO:0044454 | Nuclear chromosome part                           | 15    | 3.03           | 7.53E-03             |
| GO:0005811 | Lipid particle                                    | 6     | 7.70           | 1.34E-02             |
phenotypes may be attributable to indirect effects of maternal bacterial exposure. Exposure to various pathogens has been shown either to decrease (e.g. [37–41]) or to not affect (e.g. [42, 43]) fecundity in mosquitoes, but the effects of parental bacterial exposure on developmental time and longevity of mosquito offspring remain unclear. In \textit{Drosophila melanogaster}, infection of females with bacteria has been shown to cause reduced longevity of offspring, though these effects varied between genetic lines [44]. In \textit{Tenebrio molitor}, induction of an immune response in females has been shown to cause longer development time in their larval offspring, and in \textit{Trichoplusia ni}, parental dietary exposure to bacteria caused decreased survival and prolonged development time in offspring [45, 46]. Exposure to Csp\textsubscript{P} elicits an immune response in adult \textit{An. gambiae} [17]. It is possible that the increased larval mortality and delayed development we observed in offspring

Fig. 7 Exposure to multiple \textit{Chromobacterium} species affects mortality of \textit{An. gambiae} females. Five \textit{Chromobacterium} species were cultured under biofilm conditions for 72 h and the biofilm and culture media (i.e. biofilm supernatant) were harvested and an aliquot was filtered through a 0.2 \(\mu\)m filter to remove live cells. Culture preparations, LB or PBS were mixed 1:1 with 3% sucrose and provided to \textit{An. gambiae} adult females for 24 h. Experimental sugar meals were then removed, and all treatments were provided 10% sucrose \textit{ad libitum}. Survival was monitored for seven days. The experiment was repeated three independent times, with 20–25 individuals per treatment per replicate. Only one PBS and LB control dataset was collected, and these data are repeated on each panel to allow visualization of each individual species. Contrasts between each treatment and the appropriate control (PBS for biofilm, LB for biofilm supernatant) were conducted using Log-Rank tests followed by a Bonferroni correction.
of C.sp_P-exposed mothers is a transgenerational cost of pathogenic infection or of mounting an immune response.

In investigating the effects of exposure to cell-free C. sp_P biofilm supernatant on the adult female transcriptome, we found that genes involved in detoxification and response to insecticide exposure were significantly enriched. Exposure to insecticides has been shown to have intragenerational and transgenerational effects in mosquitoes and other insects, though the nature of these effects is diverse. For example, exposure of An. stephensi larvae to organophosphate and carbamate insecticides caused decreased fertility but increased longevity in adults [47]. Treatment of Ae. aegypti larvae with pyrethroid and organophosphate insecticides as well as a botanical extract shortened development time, increased pupal mortality and reduced longevity, and is regulated in part by quorum sensing [21, 35, 59]. As such, it is a clear candidate for causing C.sp_P-induced mosquito mortality. We found that cell-free C.sp_P biofilm supernatant does produce hydrogen cyanide at approximately 0.039 mg/l. This means that the 1 ml sugar meal provided to adult females contained a total of approximately 0.02 μg HCN, of which only a fraction would be ingested by each individual mosquito. With the exception of insects that feed on cyanogenic plant species (and have therefore evolved extremely high tolerance to cyanide) [60–62], there is very little information available regarding the susceptibility of terrestrial invertebrates to ingestion of cyanide. Fumigation studies of the wheat weevil, Sitophilus granarius, showed that exposure to 28.6 mg/l HCN induced LC50 after an 8 min exposure, and 8 mg/l HCN induced LC50 after 4 h [63, 64]. Given that these levels are orders of magnitude higher than the concentration of HCN in C.sp_P biofilm, we consider it unlikely that HCN is the source of mosquitocidal activity. Consistent with this, we found that C.sp_P biofilm supernatant caused significant mortality compared to the LB control regardless of whether it was vacuum centrifuged. Were HCN the cause of mosquito mortality, we would have expected it (and the mosquitocidal activity) to be lost after vacuum centrifugation, given that HCN is a gas and boils close to room temperature (25.6 °C). Interestingly, vacuum centrifugation itself caused a significant reduction in survival that was consistent in both treatments (C.sp_P and LB control), suggesting the presence of volatile compounds in LB that improve longevity of the adult mosquito.

In addition to exploring the nature of the mosquitocidal activity, we also investigated how exposure to C.sp_P impacts the transcriptome of adult female An. gambiae. We found that genes related to xenobiotic and insecticide detoxification were upregulated after oral exposure to cell-free C.sp_P biofilm supernatant. These genes included many cytochrome P450 genes, specifically those in the CYP6M, CYP6P, CYP6Y and CYP6Z subfamilies.
Genes from the CYP6M, CYP6P and CYP6Z subfamilies have all been shown to play a role in metabolism of (and resistance to) multiple classes of insecticides [65–67]. These findings suggest that the Csp_P biofilm supernatant contains compound(s) that evoke a physiological response in the mosquito similar to that mounted in response to insecticide exposure. It will be valuable in the future to further investigate the nature of the mosquitocidal compounds produced by Csp_P to determine whether they differ from known classes of insecticides.

We also found that exposure to cell-free Csp_P biofilm supernatant resulted in changes in transcript abundance in genes involved in nucleosome and chromatin formation. Specifically, multiple genes that encode for histone proteins were downregulated. Histone proteins form multimeric complexes, around which DNA is wound to form nucleosomes, which then pack together to form chromatin. Histone gene mRNA levels decrease naturally following DNA replication and artificially after treatment with ionizing radiation or drugs that cause DNA damage or stalled DNA replication [68–70]. This suggests that Csp_P may produce a factor that induces this state in the mosquito midgut.

In testing other Chromobacterium species, we found that all five tested had significant mosquitocidal activity when live bacterial preparations were fed to adult female An. gambiae. After removal of live bacterial cells, Csp_P, C. subtsugae and C. vaccinii retained mosquitocidal activity while C. violaceum and C. aquaticum did not. However, the mosquitocidal activity produced by Csp_P was strongest among all species tested, both before and after removal of live cells. One possible interpretation of these results is that mosquitocidal factor(s) are produced by all members of the genus, but only secreted in lethal concentrations by Csp_P, C. subtsugae and C. vaccinii. Csp_P’s especially robust activity may be due to high production of these mosquitocidal factors, or production of a unique factor not made by the other species in the genus. Chromobacterium violaceum and C. aquaticum may produce a different mosquitocidal compound that is not secreted, or accumulation in the media of the mosquitocidal factor(s) may be temporally dynamic and we may have failed to detect it in our experiment. Alternatively, live C. violaceum and C. aquaticum may kill mosquitoes by causing a lethal infection. Chromobacterium subtsugae is known to have insecticidal activity and is currently being used as a biopesticide marketed as Grandevo® (Marrone Bio Innovations) [20, 36]. Chromobacterium vaccinii has also been shown to cause mortality in moths and mosquito larvae, though the mechanism by which either of these species cause insect mortality is currently unknown [21]. Our data also show that, in addition to causing mortality in An. gambiae and Ae. aegypti [17], Csp_P is active against An. stephensi (a major vector of Plasmodium in Asia), Ae. albopictus (a vector of dengue, Zika, and chikungunya viruses), and Culex quinquefasciatus (a vector of West Nile virus). This suggests that the mosquitocidal factor produced by Csp_P generally affects mosquitoes and could be potentially used against a diverse range of mosquito species and possibly other types of insects. Chromobacterium subtsugae is very broad in its insecticidal effects, causing mortality in beetles, moths, stinkbugs and whiteflies [20]. Interestingly, C. subtsugae does not cause mortality in Culex pipiens mosquito larvae, while C. vaccinii and C. sp_P do cause mortality in Aedes aegypti mosquito larvae [17, 20, 21]. These data suggest there may be multiple compounds produced by these species that have the potential for broad-spectrum mosquitocidal activity.

Conclusions

Our results show that oral exposure to Csp_P induces significant mortality in a broad range of disease vector mosquitoes, and non-lethal exposure of adult females causes increased mortality and slower development in F1 offspring. Chromobacteria are known to produce hydrogen cyanide, but our data suggest that this is not the cause of Csp_P-induced mosquito mortality. Mosquitocidal activity persists after removal of live bacterial cells from Csp_P biofilm culture media, and oral exposure to this Csp_P treated media elicits changes in the mosquito midgut transcriptome that are similar to those that occur after exposure to insecticidal compounds and other xenobiotics. Finally, other Chromobacterium species also cause increased mortality in adult An. gambiae suggesting the Chromobacterium genus holds potential for the exploration of novel mosquitocidal compounds. That the mosquitocidal factor(s) can be isolated in cell-free preparations renders them amenable to further biochemical study to determine mode of action and increases their potential for use as chemical insecticides.

Additional files

**Additional file 1:** Figure S1. Proportion of inseminated females exposed to each bacterial treatment. Insemination status of females from each group was assessed and found to not differ. Data were collected over 3–4 replicates. Effect of treatment on insemination status was evaluated using a Kruskal Wallis test in R (χ² = 4.77, df = 2, P = 0.092). (TIFF 208 kb)

**Additional file 2:** Figure S2. Treatment of adult An. gambiae females with Csp_P does not result in increased bacterial load in breeding water of larval offspring. Two 1 ml water samples were taken from the oviposition cups and from larval trays prior to adding food or larva (baseline), and then again at 4 and 8 days after transfer of the larvae. Although the load of cultivable bacteria differed across time (F(4, 38) = 3.88, df = 3, P = 0.001), the mean bacterial load was not significantly different between the two groups F(4, 38) = 0.04, df = 1, P = 0.842 and this was consistent across time (time x treatment interaction: F(4, 38) = 0.13, df = 3, P = 0.944). Each data point represents the average CFU of cultivable bacteria for each of the three experimental replicates; error bars represent 95% confidence intervals. A two-way ANOVA was used to analyze the data. (TIFF 245 kb)
Additional file 3: Raw data for Figs. 1–7. (XLSX 185 kb)
 Additional file 4: Figure S3. Chromobacterium species biofilm harbors more bacteria than supernatant. Each species was cultured under biofilm conditions and CFU/ml were estimated from biofilm and biofilm supernatant fractions of each species. A two-factor ANOVA including species and culture fraction as factors revealed no interaction between the factors (F(4, 18) = 1.08, df = 4, P = 0.394), and there was a significant main effect of culture fraction (F(2, 22) = 11.26, df = 1, P = 0.0029) but not of species (F(4, 22) = 0.23, df = 4, P = 0.92). (TIFF 569 kb)

Abbreviations
Csp_P: Chromobacterium species Panama; PBS: phosphate-buffered saline; BEI Resources: Biodefense and Emerging Infections Research Resources Repository; ATCC: American Type Culture Collection; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen; LB: lysogeny broth; CFU: Colony Forming Units; HCN: hydrogen cyanide

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Availability of data and materials
All data analyzed in this manuscript are available in Additional file 3, with the exception of raw transcriptome data, which are available in the Gene Expression Omnibus, series entry GSE109727.

Authors’ contributions
SMS designed and conducted experiments, analyzed data, and wrote the manuscript. SVT designed and conducted experiments, analyzed data, and wrote the manuscript. BS conducted experiments and analyzed data, YD designed experiments and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Adult mosquitoes were blood-fed on mice for colony maintenance according to a protocol approved by the Animal Care and Use Committee of the Johns Hopkins University (permit number M01SH144). For blood-feeding using membrane feeders, commercial human blood from anonymous donors was used, and informed consent was therefore not applicable. This protocol has been approved by the Johns Hopkins School of Public Health Ethics Committee.

Consent for publication
Not applicable.

Competing interests
SMS and GD have filed a patent application related to this work, Publication Compositions and Methods useful for the prevention of malaria and dengue virus transmission.

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Reference
1. Brady OJ, Gething PW, Bhattacharya P, Craig M, Reynolds M, Bisanzio D, et al. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis. 2012;6:e1763.

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References
1. Brady OJ, Gething PW, Bhattacharya P, Craig M, Reynolds M, Bisanzio D, et al. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis. 2012;6:e1763.
2. Bhattacharya P, Craig M, Reynolds M, Bisanzio D, Morgan SI, Hay SI, et al. The global distribution and burden of dengue. Nature. 2013;496:504–7.
3. Mekyska HC, Matranga CB, Wohl S, Schaffner SF, Freije CA, Winnicki SM, et al. Zika virus evolution and spread in the Americas. Nature. 2015;526:411–5.
4. WHO. World Malaria Report. World Health Organization; 2016. http:// apps.who.int/iris/bitstream/10665/253038/1/9789241511711-eng.pdf?ua=1. Accessed 25 Sept 2017.
5. Bhattacharya P, Weiss DJ, Cameron E, Banianda D, Bally M, Nalayi M, et al. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature. 2015;526:207–11.
6. Vlule JM, Beach MA, Atieli FK, Roberts JM, Mount DL, Mwangi RW. Reduced susceptibility of Anopheles gambiae to permethrin associated with the use of permethrin impregnated bednets and curtains in Kenya. Med Vet Entomol. 1994;8:71–5.
7. Enayati AA, Vatanpour H, Ladoni M, Townson H, Hemingway J. Molecular evidence for a kdr-like pyrethroid resistance mechanism in the mosquito vector Anopheles stephensi. Med Vet Entomol. 2003;17:38–44.
8. Cooon KL, Brown MR, Strand MR. Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. Mol Ecol. 2016;25:5806–26.
9. Cooon KL, Vogel KJ, Brown MR, Strand MR. Mosquitoes rely on their gut microbiota for development. Mol Ecol. 2014;23:2727–39.
10. Gimonneau G, Schioffio MT, Abate L, Boissière A, Awono-Ambené PH, Nsongo SE, et al. Composition of Anopheles coluzzii and Anopheles gambiae microbiota from larval to adult stages. Infect Genet Evol. 2014;28:715–24.
11. Minard G, Mavingui P, Moro CV. Diversity and function of bacterial microbiota in the mosquito holobiont. Parasit Vectors. 2013;6:146.
12. Gendrin M, Christophides GK. The Anopheles mosquito microbiota and their impact on pathogen transmission. In: Mangun S, editor. Anopheles mosquitoes - New insights into malaria vectors. Rijeka: InTech; 2013.
13. Segata N, Baldo F, Pompon J, Garrett WS, Truong DT, Dabre RK, et al. The reproductive tracts of two malaria vectors are populated by a core microbiome and by gender- and swarm-enriched microbial biomarkers. Sci Rep. 2016;6:24207.
14. Sharma P, Sharma S, Mauya RK, Das DT, Thomas T, Lata S, et al. Sallivary glands harbor more diverse microbial communities than gut in Anopheles culicifacies. Parasit Vectors. 2014;7:235.
15. Wang Y, Gilbert BM, Kukutla P, Yan G, Xu J, Leulier F. Dynamic gut microbiome across life history of the malaria mosquito Anopheles gambiae in Kenya. PLoS One. 2011;6:e24767.
16. Guzman DS, Santos AV, Marini DC, Russo ÉDS, Peixoto AMD, Bacci M, et al. First isolation of microorganisms from the gut diverticulum of Aedes aegypti (Diptera: Culicidae): new perspectives for an insect-bacteria association. Mem Inst Oswaldo Cruz. 2007;102:919–22.
17. Ramirez JL, Short SM, Bahia AC, Sairava RG, Dong Y, Kang S, et al. Chromobacterium Csp_P reduces malaria and dengue infection in vector mosquitoes and has entomopathogenic and in vitro anti-pathogen activities. PLoS Pathog. 2014;10:e1004398.
18. Brazilian National Genome Project Consortium. The complete genome sequence of Chromobacterium violaceum reveals remarkable and exploitable bacterial adaptability. Proc Natl Acad Sci USA. 2003;100:11660–5.
19. Chi TD, Lee JC, Pandey BD, Yoo K, Jeong J. Bioleaching of gold and copper from waste mobile phone PCBs by using a cyanogenic bacterium. Miner Eng. 2011;24:1219–22.
20. Martin PAW, Gundersen-Rindal D, Blackburn M, Buyer J. Chromobacterium subtsugae sp. nov., a betaproteobacterium toxic to Colorado potato beetle and other insect pests. Int J Syst Evol Microbiol. 2007;57:993–9.
21. Martin PA, Soby S. Insecticidal strains of Chromobacterium vaccinii sp. nov. for control of insects. Patent number US 9395039 B1. 2016.
22. Ramirez JL, Souza-Neto J, Cosme RT, Rovira J, Ortiz A, Pascale JM, et al. Reciprocal tripartite interactions between the Aedes aegypti midgut...
microbiota, innate immune system and dengue virus influences vector competence. PLoS Negl Trop Dis. 2012;6:e1561.

23. Cirimotich CM, Dong Y, Clayton AM, Tubau JF, Grace PS, Reiter PJ. The combined effects of infection and temperature on dengue virus transmission by Aedes aegypti. PLoS Negl Trop Dis. 2011;5:e571.

24. Xi Z, Das S, Garver L, Dimopoulos G. Protocol for Plasmodium falciparum infection in mosquitoes and infection phenotype determination. J Vis Exp. 2007;5:e222.

25. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991;173:697–703.

26. Blumberg BJ, Trop S, Das S, Dimopoulos G. Bacteria- and IMD pathway-independent immune defenses against Plasmodium falciparum in Anopheles gambiæ. PLoS One. 2013;8:e72130.

27. Dong Y, Manfredini F, Dimopoulos G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog. 2009;5:e1000423.

28. Short SM, Morgenst EF, Macleod HJ, Talawy OAC, Dimopoulos G. Amino acid metabolic signaling influences Aedes aegypti midgut microbiome variability. PLoS Negl Trop Dis. 2017;11:e0005677.

29. Ritchie ME, Pipرحon B, Wu D, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43:e47.

30. Ritchie ME, Silver J, Oshlack A, Holmes M, Dyagama D, Holloway A, et al. A comparison of background correction methods for two-colour microarrays. Bioinformatics. 2007;23:2709–7.

31. Smyth GK. Speed, normalization of cDNA microarray data. Methods. 2003;31:626–73.

32. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37:1–13.

33. R Core Team. R: A language and environment for statistical computing. Vienna, Austria. https://www.R-project.org: R Foundation for Statistical Computing. 2017.

34. R Core Team. R: A language and environment for statistical computing. Vienna, Austria. https://www.R-project.org: R Foundation for Statistical Computing. 2017.

35. Michaelis R, Corpe WA. Cyanide formation by Chromobacterium violaceum. J Bacteriol. 1965;88:106–12.

36. Blackburn MB, Sparks ME, Gundersen-Rindal DE. The genome of the insecticidal Chromobacterium subturgidum PRAA4-1 and its comparison with that of Chromobacterium violaceum ATCC 12472. Genom Data. 2016;101:1–3.

37. Hogg JC, Hurd H. Malaria-induced reduction of fecundity during the first gonotrophic cycle of Anopheles stephensi mosquitoes. Med Vet Entomol. 1995;9:176–80.

38. Hogg JC, Hurd H. The effects of natural Plasmodium falciparum infection on the fecundity and mortality of Anopheles gambiae s.l. in north eastern Tanzania. Parasitology. 1997;114:325–31.

39. Hacker C. The differential effect of Plasmodium gallinacium and dengue virus impact on life history traits in Anopheles stephensi. Parasitology. 2013;140:151–60.

40. Variability. PLoS Negl Trop Dis. 2017;11:e0005677.

41. Freitak D, Heckel DG, Vogel H. Dietary-dependent trans-generational immune priming for offspring in an insect. Proc R Soc B Biol Sci. 2009;276:2617–24.

42. Zanchi C, Troussard J-PP, Martinaud G, Moreau J, Morer Y. Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. J Anim Ecol. 2011;80:1174–83.

43. Sanil D, Shetty NJ. The influence of sublethal exposure to temephos and propoxur on reproductive fitness and its influence on circadian rhythms of pupation and adult emergence in Anopheles stephensi Liston - a malaria vector. Parasitol Res. 2012;111:425–32.

44. Shaalan EA, Canyon DV, Younes MWF, Abdel-Wahab H, Mansour A-H. Effects of sub-lethal concentrations of synthetic insecticides and Callinis glucopcytyl diet extracts on the development of Aedes aegypti. J Vector Ecol. 2005;30:295–8.

45. Robert LL, Olson JK. Effects of sublethal dosages of insecticides on Culex quinquefasciatus. J Am Mosq Control Assoc. 1989;5:239–46.

46. Rehan A, Freed S. Fitness Cost of methoxyfenozide and the effects of its sublethal doses on development, reproduction, and survival of Spodoptera litura (Fabricius) (Lepidoptera: Noctuidae). Neotrop Entomol. 2015;44:513–20.

47. Palkary H, Enkegaard A. Sublethal and transgenerational effects of abamectin on the biological performance of the predatory thrips Scolothrips longicornis (Thysanoptera: Thripidae). J Econ Entomol. 2015;108:559–69.

48. Chen X, Ma K, Li F, Liang P, Liu YU, Guo T, et al. Sublethal and transgenerational effects of sulfonf BA on the biological traits of the cotton aphid, Aphis gossypii Glover (Hemiptera: Aphididae). Ecotoxicology. 2016;25:1841–8.

49. Bin YH, Li JH, Liu YQ, Cui L, Li YH, Xu XY, et al. Lethal, sublethal and transgenerational effects of the novel chiral neonicotinoid pesticide cyprodial on demographic and behavioral traits of Aphis gossypii (Hemiptera: Aphididae). Insect Sci. 2017;24:74–7.

50. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. 1998;280:295–8.

51. Flemming H-C, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010;8:632–33.

52. Lawrence JR, Korber DR, Hoyle BD, Costerton JW, Caldwell DE. Optical sectioning of microbial biofilms. J Bacteriol. 1991;173:6558–67.

53. Pitzen M, Flego D, Heinlheimo R, Palva ET. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen Erwinia carotovora. EMBO J. 1993;12:667–76.

54. Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, et al. Attenuation of Pseudomonas aeruginosa virulence by quorum-sensing inhibitors. EMBO J. 2003;22(15):3803.

55. Pessi G, Haas D. Transcriptional control of the hydrogen cyanide biosynthetic genes hcnABC by the anaerobic regulator AnaR and the quorum-sensing regulators LsrR and RhlR in Pseudomonas aeruginosa: J Bacteriol. 2000;182:6940–9.

56. Van Ohlen M, Herfurth AM, Kerbsdiet H, Witschott U. Cyanide detoxification in an insect herbivore: molecular identification of β-cyanoalanine synthases from Pieris rapae. Insect Biochem Mol Biol. 2016;70:699–110.

57. Wuybou N, Dermauw W, Tinly L, Stevens C, Grbic M, Fryeiresen R, et al. A gene horizontally transferred from bacteria protects arthropods from host plant cyanide poisoning. eLife. 2016;5:e2365.

58. Meyers DM, Ahmad S. Link between I-3-cyanoalanine synthase activity and differential cyanide sensitivity of insects. Biochim Biophys Acta. 1991;107:195–19.

59. Towill LE, Druy JS, Whitfield BL, Lewis EB, Galyan EL. Reviews of the environmental effects of pollutants: V. Cyanide. Washington, D.C.: U.S. Environmental Protection Agency; 1978. EPA/600/1-78/027 (NITS PB899290).

60. Bond EI. The action of fumigants on insects IV. The effects of oxygen on the toxicity of fumigants to insects. Can J Biochem Physiol. 1963;41:993–1004.

61. Edi CV, Djojbenou L, Jenkins AM, Regna K, Muskavitch MAT, Poupardin R, et al. PYP6 P450 Enzymes and ACE-1 duplication produce extreme and multiple insecticide resistance in the malaria mosquito Anopheles gambiae. PLoS Genet. 2014;10:e1004326.

62. Djojbenou AF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, et al. Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from southern Benin and Nigeria. BMC Genomics. 2008;9:538.

63. Chiu TL, Wen Z, Rupasinge SG, Schuler MA. Comparative molecular modeling of Anopheles gambiae CYP6Z1, a mosquito P450 capable of metabolizing DDT. Proc Natl Acad Sci USA. 2008;105:8855–60.

64. Graves RA, Zaduff WF. Rapid reversible changes in the rate of histone gene transcription and histone mRNA levels in mouse myeloma cells. Mol Cell Biol. 1984;4:351–7.

65. Su C, Gao G, Schneider S, Helt C, Weiss C, OReilly MA, et al. DNA damage induces downregulation of histone gene expression through the G1 checkpoint pathway. EMBO J. 2004;23:1113–35.

66. Rattray AMJ, Müller B. The control of histone gene expression. Biochem Soc Trans. 2012;40:880–5.