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# Intra-specific diversity of *Serratia marcescens* in *Anopheles* mosquito midgut defines *Plasmodium* transmission capacity

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**A critical stage in malaria transmission occurs in the *Anopheles* mosquito midgut, when the malaria parasite, *Plasmodium*, ingested with blood, first makes contact with the gut epithelial surface. To understand the response mechanisms within the midgut environment, including those influenced by resident microbiota against *Plasmodium*, we focus on a midgut bacteria species' intra-specific variation that confers diversity to the mosquito's competency for malaria transmission. *Serratia marcescens* isolated from either laboratory-reared mosquitoes or wild populations in Burkina Faso shows great phenotypic variation in its cellular and structural features. Importantly, this variation is directly correlated with its ability to inhibit *Plasmodium* development within the mosquito midgut. Furthermore, this anti-*Plasmodium* function conferred by *Serratia marcescens* requires increased expression of the flagellum biosynthetic pathway that is modulated by the motility master regulatory operon, *flhDC*. These findings point to new strategies for controlling malaria through genetic manipulation of midgut bacteria within the mosquito.**

T he transmission cycle of the malaria parasite in vector *Anopheles* mosquitoes begins with ingestion of a blood meal containing infective *Plasmodium* gametocytes. The malaria parasite undergoes substantial stage-specific losses during development within the midgut, to a level whereby in some cases complete refractoriness by the mosquito against the malaria parasites can be seen. Indeed, despite a large number of gametocytes transferred into the mosquito midgut via the blood meal, typically only a few ookinetes (<5) survive to produce oocysts after traversing the midgut wall. Accordingly, several trials of mosquito-based malaria control have been to interfere with parasite transmission mechanisms within mosquitoes[1]. While previous reports have demonstrated applicability of using genetic transformation to develop *Anopheles* mosquitoes impaired in parasite transmission[2–4], previous findings showing decreased genetic fitness of transgenic mosquitoes points to difficulties in fixing transgenes in populations[5]. Consequently, interest has turned to the use of microorganisms as biological control agents for vector mosquitoes[6,7].

Vector competence for the development of *Plasmodium* parasites within the mosquito midgut is affected by a variety of intrinsic factors[8,9]; in particular, the midgut microbiota have been reported to be important in modulating *Plasmodium* infection[10,11]. A particular species of Gram-negative bacteria suppressed *P. falciparum* oocyst formation more effectively than Gram-positive bacteria in the major African and Asian malaria vectors, *An. gambiae*, *An. stephensi*[12,13]. The mosquito's innate immune and other cellular responses to the *Plasmodium* parasite was revealed to be mediated by such microbiota by inter-species comparisons of each bacteria's ability to inhibit *Plasmodium* development[9,14–18].

Bacterial colonization of the intestine is achieved upon indefinite persistence of a population, without re-introduction of the bacterium in vertebrates. On the other hand, bacterial colonization of the mosquito midgut relies on the mosquito habitat and its feeding preference due to its holometabolous nature. The dramatic difference in habitats between larval and adult stages coupled with the inefficient transmission of bacteria from the larva to the adult stages results in a highly diverse microbiotic content[19], leading to the notion that each

bacteria species present within the mosquito midgut likely contains high phenotypic plasticity. Whether the resulting intra-species variation can influence malaria transmission through the mosquito midgut remains unknown. Here we focus on the intra-specific diversity of the midgut bacterial species *Serratia marcescens* and showed that phenotypic diversity of this bacteria's cellular and structural features was regulated by *flhDC* operon and correlated with its ability to modulate the *Anopheles* mosquito's vector competency for *Plasmodium* parasite transmission.

## Results

***Serratia marcescens* reduces the *Plasmodium* parasite load in *Anopheles* mosquitoes.** In nature, the mosquito midgut contains diverse communities of commensal microorganisms. To develop a microbiota-resource to understand bacterial diversity within the mosquito midgut, the bacteria isolated from the midguts of laboratory-reared, adult *An. stephensi* were identified by 16S ribosomal RNA (rRNA) sequencing. Of the isolated bacteria, *Serratia marcescens* (strain HB3) appeared to be the primary species (Tables S1, S2). It

was not surprising considering that bacteria of the genus *Serratia* are ubiquitous in nature including inside of disease vector species[20–24].

To investigate potential influence of *S. marcescens* on *Plasmodium* development within the mosquito, we prepared aseptic mosquitoes through antibiotics delivered during sugar feeding[15] (Fig. S1B). Upon challenge with *P. berghei* these aseptic mosquitoes were found to be more susceptible to *P. berghei* infection compared to naïve mosquitoes (Fig. 1A and data not shown). When *S. marcescens* HB3, transformed with a streptomycin-resistance plasmid, was reintroduced into the aseptic mosquitoes through feeding, this bacteria was cleared quickly from the mosquito to such a degree whereby no mosquitoes contained detectable *S. marcescens* HB3 in the midgut 3 days after bacterial feeding (Fig. S1A). Thus, in the following infection studies, mosquitoes were allowed to feed continuously on a solution containing *S. marcescens* HB3 (Fig. S1B, C). Consistent with a previously reported negative influence of Gram-negative bacteria on *Plasmodium* development[10–13,15], *S. marcescens* HB3 inhibited *P. berghei* oocyst formation in *An. stephensi* (Fig. 1A); nearly 10-fold more oocysts developed in aseptic mosquitoes than those containing live
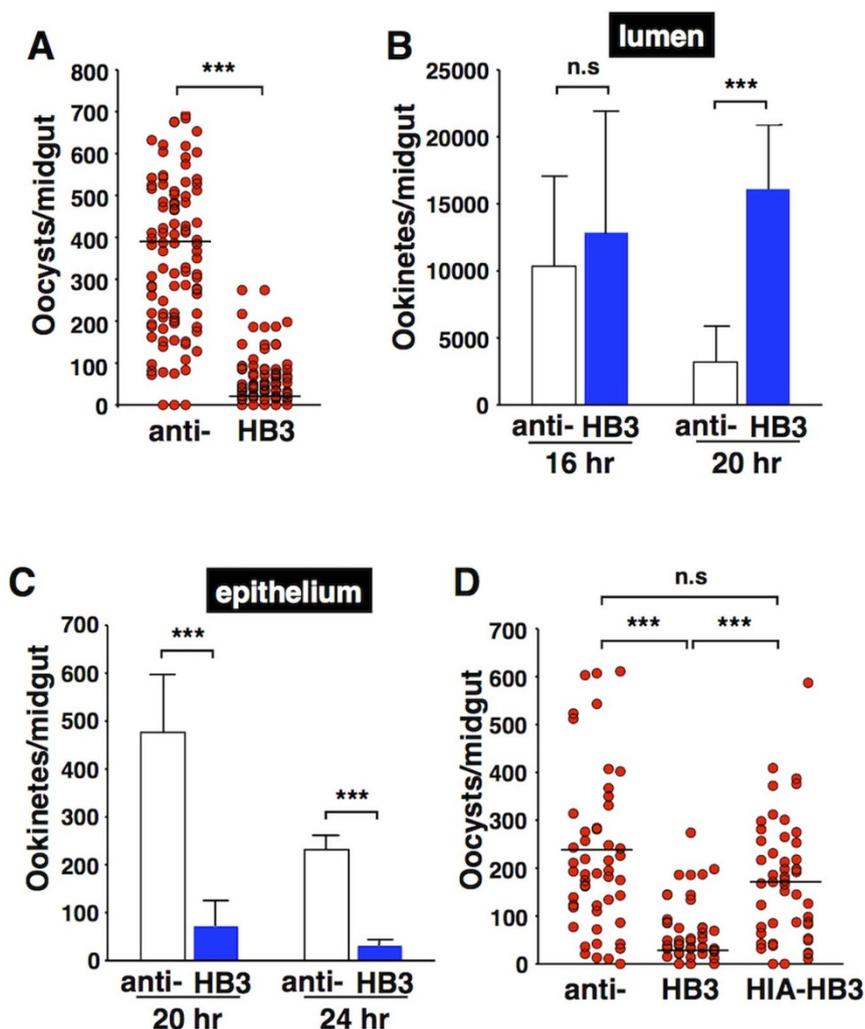


**Figure 1 | *Serratia marcescens* reduces the *Plasmodium* parasite load in *Anopheles* mosquitos.** (A) *P. berghei* oocyst counts in the midgut 8 days after *An. stephensi* mosquitoes were fed on a *P. berghei*-infected mouse. Circles (red) represent the number of parasites from an individual mosquito, and horizontal lines indicate the median. ***$p < 0.001$, Mann-Whitney test. (n = 100/group). (B and C) *P. berghei* ookinete counts in the midgut lumen 16 and 20 hours (B) and midgut epithelium 20 and 24 hours (C) after mosquitoes were fed on a *P. berghei*-infected mouse. Error bars show standard deviation. ***$p < 0.001$; n.s., not significant, unpaired *t*-test. (n = 50/group). (D) Effect of heat-inactivated *S. marcescens* HB3 on *P. berghei* load in *An. stephensi* mosquitoes. Oocyst numbers were determined as in (A). ***$p < 0.001$; n.s., not significant, Mann-Whitney test. (n = 50/group). anti-, antibiotics treatment; HB3, *S. marcescens* HB3 treatment; HIA-HB3, heat-inactivated *S. marcescens* HB3. The experiments in (A) to (D) were performed more than once with similar results.

*S. marcescens* HB3 ($p < 0.001$) compared to aseptic control mosquitoes, suggesting that *Serratia* species in the midgut lumen exert anti-*Plasmodium* effects.

**Serratia marcescens influence on mosquito susceptibility to Plasmodium parasites occurs during ookinete invasion.** Two well-known population bottlenecks in *Plasmodium*-infected mosquitoes involve ookinete invasion of the midgut and sporozoite invasion of the salivary glands[25]. To investigate the timing of the observed parasite inhibition, we monitored the development of *Plasmodium* ookinetes in the presence of *S. marcescens* HB3. No difference in numbers of ookinetes in the midgut lumen could be seen between aseptic and *S. marcescens* HB3 containing mosquitoes 16 hours after blood ingestion (Fig. 1B). Interestingly, 20 hours after blood feeding, 5-fold more ookinetes remained in the midgut lumen compared to control mosquitoes ($p < 0.001$) (Fig. 1B). The morphology of ookinetes was similar in each cohort (data not shown). In contrast, the number of ookinetes observed within the midgut epithelium was approximately 8-fold higher in aseptic mosquitoes ($p < 0.001$), compared to the bacteria-containing cohort (Fig. 1C). Taken together, these results suggest that *S. marcescens* exerts its influence on *Plasmodium* development during ookinete invasion of the mosquito midgut.

**Serratia marcescens exerts non-immune anti-Plasmodium activity.** In *Drosophila*, the NF-κB-related transcription factors Relish and Dif are involved in immune signaling through transcriptional activation of immune related genes including those encoding the antimicrobial peptides (AMPs)[26]. The Toll and IMD signaling pathways activate distinct members of the NF-κB family for combating infections by fungi and bacteria[27–29]. Previous reports also indicate that vector mosquitoes rely on NF-κB-mediated immunity to regulate both bacterial and *Plasmodium* infections[18]. As previously reported, Gram-negative bacteria activated *IMD* pathway and heat-inactivated (HIA) Gram-negative bacteria inhibited *Plasmodium* oocyst formation in mosquito[9,15]. Surprisingly, neither mosquitoes challenged with HIA *S. marcescens* HB3 or bacterial supernatant showed resistance to *Plasmodium* development (antibiotics vs. HIA-HB3, $p = 0.063$ and HB3 vs. HIA-HB3, $p < 0.001$) (Fig. 1D and data not shown), suggesting bacteria are conferring resistance to *Plasmodium* development by means not involving the innate immune response. In addition, no significant induction of the AMPs *As defensin* and *As cecropin A* was observed in *S. marcescens* HB3, HB18, or HB3 flagella-fed mosquitoes (Fig. S5). Although still another possibility is that innate immunity could serve for attacking these parasites within microenvironments such as insect gut surface with complex structure, it suggests that *S. marcescens* HB3 may confer resistance to *Plasmodium* development through means unrelated to the innate immune response.

**Phenotypic variation of Serratia marcescens affects Plasmodium inhibition.** After observing rapid clearance of *S. marcescens* HB3 from the mosquito midgut (Fig. S1A) we sought to create a new *S. marcescens* strain with cellular phenotypes that might affect both bacterial clearance rates as well as *Plasmodium* load. A previous report indicates that midgut microbiota has an important role to digestion, nutrition, and development of mosquito[30]. In the case of using an experimental procedure that compares aseptic and *S. marcescens* HB3-exposed mosquitoes, gut nutritional status modulated by microbe vital activity might influence *Plasmodium* development. To isolate a *S. marcescens* strain adapted to the mosquito midgut, unlike the rapidly eliminated HB3 (Fig. S1A), bacteria recovered from the midgut 3 days after feeding with HB3 were subjected to multiple rounds of selection *in vivo* (Fig. S2A). Accordingly, a *S. marcescens* strain, HB18, which survived in the mosquito midgut up to 18 days, was isolated (Fig. S2B-D). We next examined the capacity of *S. marcescens* HB18 to influence *Plasmodium* development

and found that HB18 completely lost the ability to inhibit *Plasmodium* development (antibiotics vs. HB18, $p = 0.105$) (Fig. 2A). Considering *S. marcescens* HB18 is derived from *S. marcescens* HB3, this result suggests that phenotypic diversity likely plays a role in *S. marcescens*' influence on *Plasmodium* development.

**S. marcescens HB18 phenotypic diversity related to flhDC operon.** Since the *S. marcescens* strain HB18 appeared to be retained within the mosquito midgut upon introduction we investigated the mechanisms behind its ability to establish a stable population. Thus we examined the cellular properties of *S. marcescens* HB18 in relation to its colonization of the midgut environment. Swarming motility is a social form of migration that, like swimming motility, is driven by rotating flagella that results in rapid movement. Swimming on a surface occurs when the fluid film is sufficiently thick and the cells move individually and randomly. On the other hands, Swarming is continuous and regularly follows the long axis of the cells, which are predominantly aggregated in bundles during their movement[31]. *S. marcescens* HB3 showed high swarming and swimming motility, whereas, HB18, an efficient midgut colonizer, was completely immobile (Fig. S3A, B). Consistent with previous findings showing that swarming cells have dramatically increased numbers of flagella on their larger overall cell surface than their swimming counterparts[32], *S. marcescens* HB3 exhibited large cell size variation with many flagella, while HB18 appeared subglobular and non-flagellar (Figs. 2B, C ($p < 0.001$), 3C).

Establishment and maintenance of the commensal microflora is a complex and multi-factorial process and some reports indicate a reciprocal interaction between the *flhDC* operon-regulated bacterial phenotypes and colonization ability of the bacteria in the intestine[33–35]. Enterobacteriaceae including the *Serratia* genus are generally motile and produce flagella, with over 50 genes involved in the process including cell division, cell differentiation, swarming/swimming motility, and virulence genes[36–41]. The single class 1 operon comprised of the *flhDC* genes, a master operon of the flagellar transcriptional cascade[42]. Mutations and deletions of *flhDC* operon are found in some types of non-motile bacteria lacking flagella, such as *Shigella*[43], therefore we examined the transcriptional level of *flhDC* in *S. marcescens* HB18. Indeed, expression of *flhDC* was found to be dramatically lower in the non-motile, non-flagellular *S. marcescens* HB18 strain (Fig. 2D). Comparison between the HB3 and HB18 strains revealed identical coding regions (data not shown). However, a single nucleotide substitution and two insertion mutations within the −35 consensus promoter element of *flhDC* of *S. marcescens* HB18 were found, likely explaining the loss of transcription seen in this strain (Fig. 3A). This data points to a genetic mechanism behind intra-specific diversity that directly influences colonization ability of a vector midgut species.

**flhDC-regulated bacterial phenotypes correlate with anti-Plasmodium function.** In order to verify that mutations identified in the promoter region of HB18 were responsible for observed reduced expression of *flhDC*, the *flhDC* coding region behind control of the HB3 promoter sequenced was expressed in *S. marcescens* HB18. Together with the increased *flhDC* mRNA expression (Fig. 3B), we also observed a restoration of cell length indicating the observed phenotypic diversity was caused by lowered *flhDC* expression (Fig. 3C–E).

Since the HB3 length phenotype was rescued upon *flhDC* expression, we examined if the HB3-conferred anti-*Plasmodium* activity was also restored. Indeed, *S. marcescens* HB18 transformed with *flhDC* plasmid recovered its ability to inhibit *Plasmodium* oocyst formation (Fig. 3F); 3.9-fold fewer oocysts developed in mosquitoes fed on *S. marcescens* HB18 (*flhDC+*) ($p < 0.001$) compared to the HB18-infected mosquitoes. Additionally, the *S. marcescens* HB18 (*flhDC+*) also reverted to the wild type phenotype in other properties that included ability to colonize the mosquito midgut and effect on
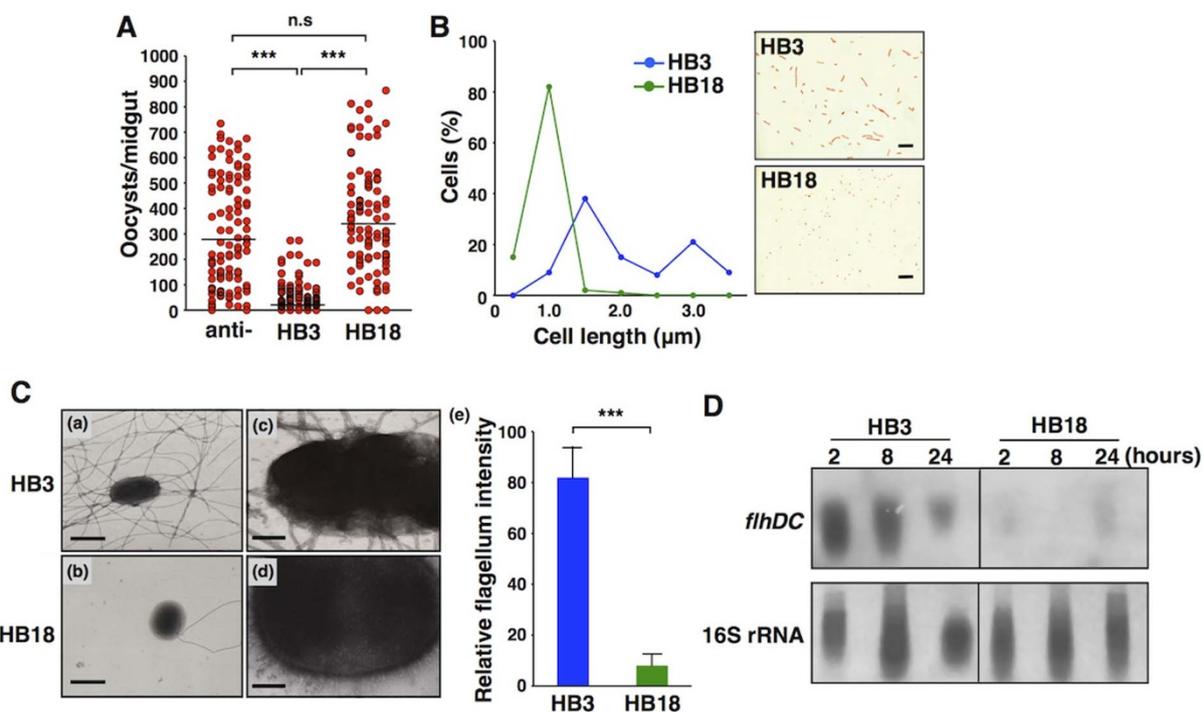
**Figure 2 | *Serratia marcescens* without phenotypic diversity loses its *Plasmodium* inhibitory ability.** (A) *P. berghei* oocyst counts on the *An. stephensi* midgut 8 days after mosquitoes fed on *P. berghei*-infected mice. Circles (red) represent the number of parasites from an individual mosquito, and horizontal lines indicate the median. ***$p < 0.001$; n.s., not significant, Mann-Whitney test. (n = 100/group). The experiment was repeated at least three times. (B) (left) Frequency distribution of the bacterial major axis lengths 10 hours after *S. marcescens* inoculation. (n = 500/group). (right) Gram-staining of *S. marcescens* HB3 and HB18 reveals their size difference. Bars = 3 μm. (C) (left) Bacterial flagella on *S. marcescens* HB3 and HB18 ((a)–(d)). *S. marcescens* HB3 contained many mature flagella. (a) and (b) Bars = 1 μm, (c) and (d) Bars = 250 nm. (right) Relative intensity of flagella per bacterium measured using Scion Imaging software ((e)). Error bars show standard deviation. (n = 208/group). ***$p < 0.001$, student's *t*-test. (D) Expression of *flhDC* genes in *S. marcescens* HB3 and HB18. *flhDC* mRNA expression levels were determined by northern blot analysis using a probe corresponding to *flhDC* 2, 8, and 24 hours after cultivation. The amounts of mRNA were confirmed using 16S ribosomal RNA. anti-, antibiotics treatment; HB3, *S. marcescens* HB3 treatment; HB18, *S. marcescens* HB18 treatment.

survival rate of mosquito (data not shown). These results suggest that a cluster of specific bacterial characters related to cellular structure and motility in *S. marcescens* is inextricably associated with the ability to inhibit *Plasmodium*.

**Intra-species variation of *S. marcescens* is associated with vectorial competency in a malaria endemic area.** To explore whether intraspecific diversity of *S. marcescens* is associated with vectorial competency of *Plasmodium*-transmitting mosquitoes in malaria endemic areas, we analyzed the microbiota in the midgut of wild mosquitoes (*An. gambiae*) collected in Burkina Faso, a country with frequent outbreaks of malaria (Table S3)[44]. Among seven Gram-negative bacterial species isolated, six strains of *S. marcescens* were identified based on their 16S rRNA sequence and conventional phenotypic identification by Gram staining, culture, and biochemical methods (Table S3). Diversity in cell length could be seen from bacteria taken from midguts of wild mosquitoes with Gd-17 length approaching that of HB3 (Fig. 4A–C). On the other hand, there was a similar bias toward shorter cells that we observed in *S. marcescens* HB18 for a subpopulation of strains (Gu-11, Gd-6, and Gd-9). In addition, the cell size of *S. marcescens* Gu-1 and Ku-3 was intermediate between HB3 and HB18 (Fig. 4A–C, compared to Fig. 3C), indicating a highly diverse bacterial length in nature. The levels of *flhDC* mRNA were equivalent in these all strains tissues (Fig. S5), suggesting that other downstream components of the *flhDC* pathway may be affected to induce those bacterial phenotypes.

These strains were tested for their ability to influence *Plasmodium* development within the mosquito midgut. As with length variation, great diversity could be seen, however, a statistical correlation

between cell length and oocyst number could be seen (Fig. 4D, E); five different strains significantly inhibited *P. berghei* oocyst development in *An. stephensi* (78.3% (Gu-1), 48.9% (Gu-11), 58.4% (Gd-6), 80.6% (Gd-17), and 67.4% (Ku-3) fewer oocysts than that of HB18) ($p < 0.01$). Significant positive correlations were found between the average number of oocysts in the midgut and the cell size diversity index ($r = 0.7449$, $p < 0.01$) or the average length of cells ($r = 0.7142$, $p < 0.01$), indicating that longer cells exhibit a more potent inhibitory effect on *Plasmodium* development. Despite the natural variation of cell length, it appears that cells tending to be longer, even in nature, exhibit potent inhibitory effects on *Plasmodium* development.

## Discussion
Effective malaria control strategies targeting vector mosquitoes currently include insecticide treatment delivered through spraying of houses or insecticide-impregnated mosquito nets. While these methods are effective at decreasing mosquito numbers, they also contribute to the rise of insecticide-resistance vector species. An alternative strategy involves inhibition of *Plasmodium* development within the mosquito. One such strategy involves release of transgenic mosquitoes resistant to *Plasmodium* that could compete with wild populations, however, transgenes have proven difficult to fix in populations. In response to this problem, symbiotic bacteria have been proposed as paratransgenic tools for control of malaria that act mainly by killing *Plasmodium* ookinete/oocysts in the mosquito midgut[11]. However, new problems associated with resistance of *Plasmodium* parasite may easily emerge during the paratransgenesis of the mosquito since the principal mode of action is to attack/kill the pathogen
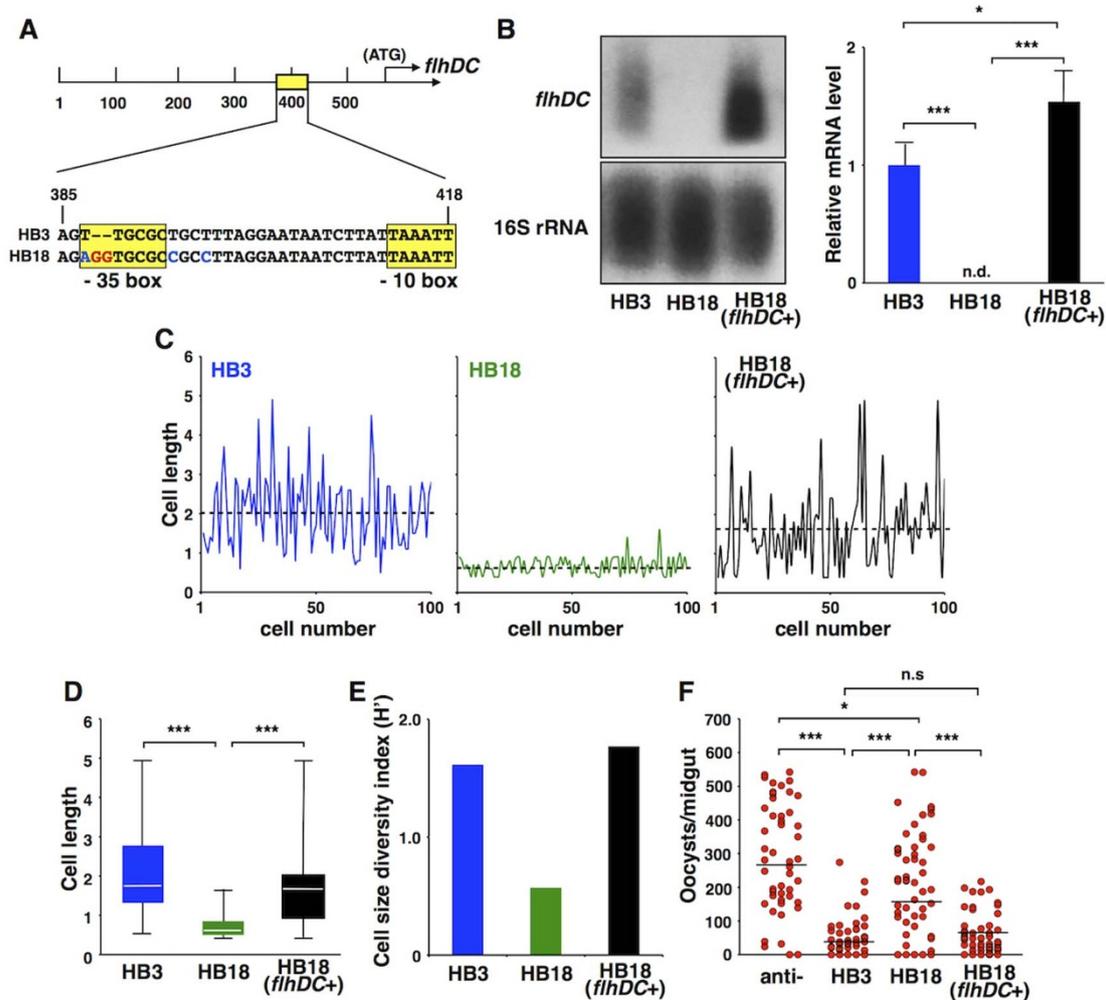
**Figure 3 | *flhDC*-regulated bacterial phenotypes correlate with their anti-*Plasmodium* function.** (A) Essential *flhDC* promoter region in *S. marcescens* HB3 and HB18. The *flhDC* genes from HB3 and HB18 were obtained by PCR with primers (flhDF2 and flhCR2) (Table S4) and sequenced from the PCR product. The *flhDC* gene (GenBank Accession Number AF077334) contains a −10 box (TAAATT; nucleotide position 413–418) and −35 box (TTGCGC; nucleotide position 387–392). The −35 box of HB18 had one nucleotide substitution (blue) and two insertions (red) compared to HB3. (B) (left) Rescued *flhDC* gene expression in HB18 (*flhDC+*). The *flhDC* mRNA levels were determined by northern blot 10 hours after cultivation, as in Fig. 2D, and confirmed using 16S ribosomal RNA. (right) Relative mRNA signal intensities measured using ImageJ (NIH). Error bars: standard deviation (n = 3/group). ***$p < 0.001$ *$p < 0.05$; n.d., not detected, student's *t*-test. (C–E) Cellular phenotypes of HB18 are caused by loss of *flhDC* expression. (C) Length of individual bacteria 10 hours after *S. marcescens* inoculation. Dashed lines indicate the mean (μm). (n = 100/group). (D) Distribution of bacterial length. ***$p < 0.001$, Mann-Whitney test. Box plots in which whiskers indicate the most distant data point no more than 2.5 times the interquartile distance from the median. (n = 100/group). (E) Cell-size diversity compared using the Shannon-Weaver diversity index (H′). (n = 50/group). (F) *P. berghei* oocysts in the *An. stephensi* midgut 8 days after feeding on *P. berghei*-infected mice. Circles (red) represent the number of parasites from an individual mosquito, and horizontal lines indicate the median. ***$p < 0.001$, *$p < 0.05$; n.s., not significant, Mann-Whitney test. (n = 50/group). anti-, antibiotics; HB3, HB18, HB18 (*flhDC+*): *S. marcescens* HB3, HB18, and HB18 (*flhDC+*) treatment, respectively. The experiment was performed at least three times with similar results.

itself. In this study, we showed that *S. marcescens* HB3 interrupted ookinete invasion through the midgut rather than by killing *Plasmodium* parasites (Fig. 1A–C) suggesting that parasite entry into the mosquito midgut epithelial cells might be an effective target stage for blocking *Plasmodium* development that can be used in combination with complementary malaria control strategies.

Toward the development of a novel paratransgenic strategy, symbiotic bacteria should possess two sustained abilities: to inhibit *Plasmodium* development and to survive within the mosquito midgut for long periods after a single introduction. Bacteria living in nature are constantly subjected to stress and respond to them phenotypically by modulating their gene expression profile[45]. In this report, we showed that a bacterial species inhabiting a constrained environment—the mosquito midgut—exhibited intra-specific

variations including conferred resistance to resist *Plasmodium* infection by the host. Because the mosquito is a holometabolous insect, bacterial colonization of its midgut is an opportunistic event, thus, diversity of *S. marcescens* phenotypes inside the mosquito midgut may depend greatly on intrinsic stresses of the enclosed environment. In fact, the anti-*Plasmodium* strain *S. marcescens* HB3 needs to be continuously introduced to the vector to exert this ability, suggesting that exclusion mechanisms in the mosquito, including its innate immunity, constrict these "prominent" bacteria populations. To obtain midgut bacteria that retain anti-*Plasmodium* function and the ability to colonize the mosquito midgut under such stress conditions, comparison analyses to determine the intra-species diversity of ubiquitous bacterial genus in nature may be an efficient method for uncovering useful
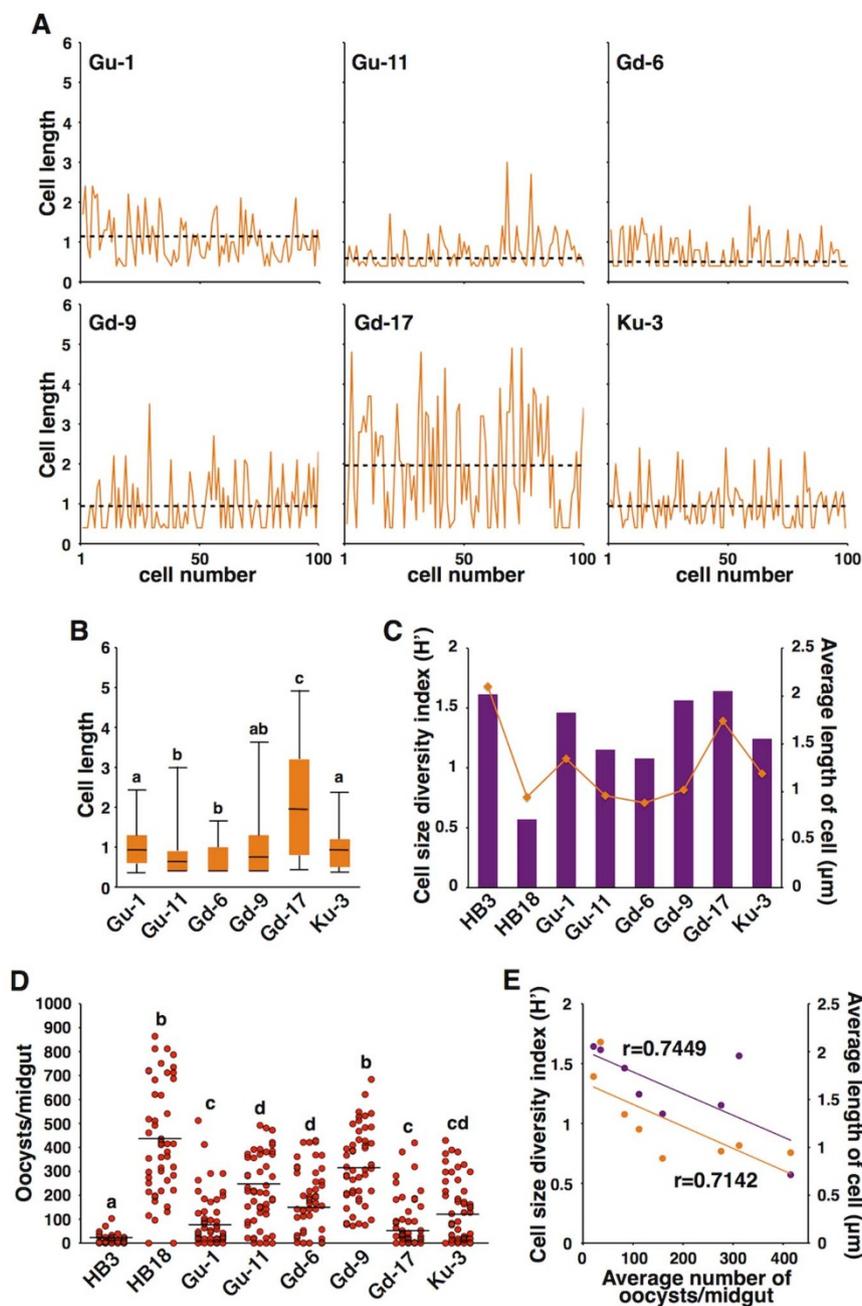
Figure 4 | **Intra-species variation of *S. marcescens* is associated with the vectorial competency of *Plasmodium*-transmitting mosquitoes in a malaria endemic area.** (A–C) *S. marcescens* from wild mosquitoes (*An. gambiae*) collected in Burkina Faso exhibit large fluctuations in cell size. (A) Length of individual bacteria 10 hours after *S. marcescens* inoculation. Dashed lines indicate the mean (µm) (n = 100/strain). (B) The distribution of bacterial lengths compared using the Mann-Whitney test (different letters signify distinct statistical groups; $p < 0.01$). Box plots were as in Fig. 3D (n = 100/strain). (C) Cell-size diversity (Purple bars) was compared using the Shannon-Weaver diversity index (H′) (n = 500/strain). Orange line chart shows average cell length (µm). (D) *P. berghei* oocysts in the *An. stephensi* midgut 8 days after feeding on *P. berghei*-infected mice. Circles (red): number of parasites from an individual mosquito. Horizontal lines: median. Distributions compared using the Mann-Whitney test (letters signify different statistical groups; $p < 0.01$) (n = 50/strain). The experiment was performed at least three times with similar results. (E) Pearson correlation analysis comparing cell-size fluctuation and anti-*Plasmodium* function. Purple: cell-size diversity index (H′) versus average number of oocysts (r = 0.7449, $p < 0.01$). Orange: average cell length (µm) versus average number of oocysts (r = 0.7142, $p < 0.01$).

candidate genes, molecules, or mechanisms (e.g., ROS) for *Plasmodium* inhibition.

In this study the *flhDC* locus was found to be central to both colonization ability and anti-*Plasmodium* activity in a converse relationship; lowered colonization ability was inversely related to anti-*Plasmodium* activity. It remains to be determined if these two processes can be separated in attempt to find strains able to colonize mosquito midguts in a single infection that would confer anti-*Plasmodium*

activity to its host. The finding of a central role for the *flhDC* locus suggests molecular mechanisms that could be manipulated in attempt to discover or make strains of commensal bacteria as effective adjuncts to current methods of malaria prevention and treatment.

## Methods

**Ethics statement.** This study was carried out in strict accordance with the recommendations in the Guide for the Laboratory Animals of the Obihiro University

of Agriculture and Veterinary Medicine and The Jikei University School of Medicine. The protocol was approved by the Committee on the Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit Number: 21–41 and 21–42) and The Jikei University School of Medicine (Permit Number: 23–020). All experiments using mice were performed under anesthesia, and all efforts were made to minimize suffering in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science and the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

**Insect maintenance.** *Anopheles stephensi* mosquitoes were maintained in the laboratory at 27°C and 80% relative humidity with a 12-hour light/dark cycle.

**Isolation of microbiota from the mosquito midgut.** Both laboratory-reared and wild mosquitoes were used to isolate and identify bacteria species in the midgut. Wild mosquitoes were collected from three different villages (Goundry, Goden, Koubri) within the Sudan-Savanna (Sudano-Sahelian) ecological zone of tropical shrubland and dry forest in Burkina Faso in November 2008. The mosquitos were collected indoors using an aspirator to avoid damaging the bacterial flora in the mosquito midgut, as previously described[48]. Blood-fed female *An. gambiae* mosquitoes were identified by their morphological characteristics. The laboratory-reared mosquitoes (*An. stephensi*) were collected from the breeding cage using an aspirator. Individual mosquitos (*An. gambiae* or *An. stephensi*) were surface-sterilized in 70% ethanol for 5–10 min, and rinsed three times in sterile phosphate-buffered saline (PBS). The midgut was isolated and homogenized in 200 μl sterile PBS. The midgut homogenate was spread on Trypticase soy agar (TSA) supplemented with 5% sheep blood, as a nonselective medium for isolating midgut bacteria, and incubated at 37°C under aerobic conditions for 24 hours. Individual bacterial colonies with distinct morphologies were subcultured for species identification both by standard biochemistry analysis and by polymerase chain reaction (PCR) amplifying the variable region of the 16S ribosomal RNA gene using universal bacterial primers 27F and 1492R. The primer sequences are listed in Table S4.

To isolate a *S. marcescens* strain well-adapted to mosquito midgut, multiple rounds of *in vivo* selection were performed as previously described[14]. Newly emerged adult mosquitoes were fed the solution containing 10% sucrose, antibiotics (penicillin 100 units/ml and streptomycin 0.1 mg/ml), and *S. marcescens* HB3. The mosquitoes were screened daily for the presence of GFP-positive *S. marcescens* and the maximum length of time that the bacteria survived in the mosquito midgut was determined. *S. marcescens* populations that persisted longer in the midgut were recovered from first round of selection as described above. These bacteria were then grown in culture and used for the second round of selection. This process was repeated six times.

**Experimental challenge of mosquito with bacteria and flagella.** To challenge *An. stephensi* with isolated *Serratia marcescens*, streptomycin-resistant *S. marcescens* strains were generated as follows. All the *Serratia* strains used in this study were innately penicillin-resistant (data not shown). For antibiotic treatment, adult mosquitoes that had newly emerged from pupae placed in a sterilized cup were fed a mixture of antibiotics (penicillin 100 units/ml and streptomycin 0.1 mg/ml) (Sigma) in 10% sucrose on filter paper. At six days post emergence, mosquitoes were starved for 12 hours before blood feeding. After blood feeding, the mosquitoes were provided daily with freshly prepared sterile 10% sucrose solution containing these antibiotics and/or bacteria as described below. The streptomycin-resistant *pUC*-based plasmid pGFPuvsm (Table S4) was electroporated into *S. marcescens* using a BioRad Gene Pulser (25 μF, 2.5 kV, and 200 Ω). *S. marcescens* transformed with the GFP marker were grown overnight at 37°C to the stationary phase. Bacterial cells from 1.5 ml of overnight culture were pelleted, rinsed three times in sterile PBS, and resuspended in 10% sucrose solution containing antibiotics (penicillin (100 units/ml) and streptomycin (0.1 mg/ml)), to a final concentration of $OD_{600 nm} = 3$. The antibiotics sterilized the mosquito midgut and allowed only the recombinant *S. marcescens* to settle and grow. To examine the number of *S. marcescens* cells in the midgut, a colony-forming assay was performed as follows. Newly emerged adult mosquitoes were fed the solution containing 10% sucrose, antibiotics, and *S. marcescens*. The mosquitoes that fed to repletion on the bacteria meal were selected and kept in a humid insectary. A group of mosquitoes was kept aside as the non-blood-fed control, and the remaining mosquitoes were given a blood meal. These mosquitoes were dissected after a certain period of time, and the midgut bacteria were isolated as described above, counted, and shown as cfu (colony-forming units). Mosquitoes that did not feed on blood were discarded.

To challenge *An. stephensi* with flagella purified from *S. marcescens*, bacterial cells from 1 L of overnight culture were pelleted, rinsed gently three times in 0.01 M phosphate buffer (pH 7.0), and mechanically deflagellated by using the Multi-Mix Homogenizer (16,000 rev/min for 60 sec). The cells were removed by centrifugation at 5,000 × g for 30 min. The cell pellet was washed once and the cells were again sedimented by centrifugation. The pooled supernatant were subjected to centrifugation at 105,000 × g for 30 min to sediment the flagella. The flagella were resuspended in 0.5 ml phosphate buffer by allowing them to stand in the refrigerator overnight and mixed in 10% sucrose containing antibiotics (penicillin (100 units/ml) and streptomycin (0.1 mg/ml)).

**Experimental challenge of mosquitoes with the *Plasmodium* parasite.** The *P. berghei* ANKA strain (containing GFP driven by the *hsp70* promoter, a gift from Dr. M. Yuda[46]) was maintained by serial passage in 7- to 8-week-old female BALB/c mice. Parasitemia was monitored microscopically by blood smears that were air-dried, methanol-fixed, and stained with 10% Giemsa solution. The parasitemia reached 3–6% and 1–2 exflagellating gametocytes/field (400×) 5–6 days after blood inoculation from infected donor mice. To examine exflagellation, 2 μl of blood was added to a microtube containing 7 μl complete ookinete medium and 1 μl heparin (1 mg/ml in PBS). Female *An. stephensi* mosquitoes were fed on anaesthetized gametocytemic mice for 1 hour at 19°C. All the treatment groups in a single experiment fed on the same mouse for equal ingestion. Blood-fed mosquitoes were kept at 19°C until dissection. To analyze the *Plasmodium* burden in challenged mosquitos, the midgut was dissected from the surviving mosquitoes, and GFP-positive oocysts were counted under a fluorescence microscope. The ookinete number in the midgut lumen was determined by counting the parasites in the midgut blood content from a mosquito 16–20 hours after blood feeding. To quantify the parasites within the midgut epithelium cells 20–34 hours after infection, the midguts were dissected in 1% paraformaldehyde, washed with PBS to remove the blood inside, and fixed in 4% paraformaldehyde for 1 hour. Immunostaining of the midgut was carried out as previously reported[47] with some modification. The following antibodies and fluorescent materials were used: rabbit anti-GFP antibody (1 : 1000, MBL) and Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1 : 100, Invitrogen), and Alexa 647-phalloidin (1 : 20, Invitrogen). All fluorescent signals were examined using a TCS SP5 confocal microscope (Leica). To analyze the topology of the midgut with *Plasmodium* ookinetes, optical sections were obtained along the z-axis at 0.2-μm intervals, and the images of the X-Z and Y-Z planes were reconstructed using Leica AF software (Leica).

**Analysis of bacterial phenotypes.** Bacterial cell sizes were compared by microscopic analysis of gram-stained cell smears. In addition, flagella formation was examined using transmission electron microscopy (TEM) and cell length was examined using scanning electron microscopy (SEM), as described previously[36,49]. To investigate the motility of *S. marcescens*, a swimming motility assay was performed on motility agar (LB medium solidified with 0.35% agar) by sterile needlepoint inoculation from an overnight culture of *S. marcescens* into the center of an agar plate surface, followed by incubation at 30°C. The swarming motility was assayed on swarming agar plates (LB medium solidified with 0.8% agar) by inoculating 3 μl of an overnight broth culture of *S. marcescens* onto the center of the agar plate surface and incubating at 30°C. The motility and migration distances were recorded hourly. To rescue the *flhDC* expression of *S. marcescens* HB18, a plasmid pGFPuvsm (*flhDC*+) was constructed as follows. A fragment (1590 bp) from the *S. marcescens* HB3 *flhDC* gene containing both the proximal promoter and the coding region was amplified using the primer pair HB16/HB17. The fragment was inserted into the *Spe* I/*Apa* I site of pGFPuvsm to generate pGFPuvsm (*flhDC*+), which was electroporated into *S. marcescens* HB18 using a BioRad Gene Pulser (25 μF, 2.5 kV, and 200 Ω).

**Invasive challenge of mosquito with bacteria.** Microbial challenge was performed as previously described[47]. Before injection, the *S. marcescens*-containing medium was adjusted to the appropriate concentration (0.01 OD for mosquito) with culture medium using Gene Quant pro (Amersham). Female *An. stephensi* were anesthetized with CO_2 and injected with each strain of bacteria in 65 nl of medium. Injection was carried out using an individually calibrated pulled glass needle attached to an IM-300 microinjector (Narishige). Mosquitoes were injected in the abdomen, close to the junction with the thorax and just ventral to the junction between the ventral and dorsal cuticles. After injection, the insects were transferred to fresh cages.

**Northern blot analysis.** Two different probes corresponding to *flhDC* were generated by PCR using primers (flhD-PF and flhD-PR for *flhDC*) (Table S4) and used for Northern analysis to target the *flhDC* mRNA molecules. Total RNAs were isolated from *S. marcescens*, using Max Bacterial Enhancement Reagent (Invitrogen). Northern blotting was performed as previously described[50]. The *flhDC* expression was analyzed with the STORM Bioimager (Amersham) and quantified with the ImageJ software (NIH).

**Quantitative real-time PCR.** Mosquito samples were prepared by the same methods for experimental challenge of mosquito with bacteria. Total RNA was extracted from 70–80 dissected mosquito midguts. DNA removal and total RNA isolation was performed with the TRIzol (Life Technologies) as per manufacturer's instructions. Reverse transcription and PCR reactions were performed on an ABI StepOne Plus (Applied Biosystems) using the Taqman RNA-to-CT 1-Step Kit (Applied Biosystems), following the manufacturer's instructions and with the following cycle conditions: 48°C–15 min, 95°C–10 min; 40 cycles of: 95°C–15 s, 60°C–1 min. Primers were as follows: *As cecropin A*, forward 5′-GTG TGT TCA AGG CAG CTG AGA A-3′, TaqMan probe 5′-CTC TCC CGG TGG TGG CAG G-3′, reverse 5′-TCT CTT CAT CCA AGA GCC TTG AC-3′; *As defensin*, forward 5′-GTC GGC AGC AGC CTT TGT-3′, TaqMan probe 5′-CCG CGC ATT GCA TCG CTC G-3′, reverse 5′-TTG CAG TAT CCG CCA CGA T-3′. Real-time PCR was monitored and analyzed with StepOne Software version 2.1 (Applied Biosystems). Relative quantitations were assigned based on a standard curve generated by serial dilution of RNA from *S. marcescens* HB3-injected mosquitos. All samples were run in quadruplicate and AMP relative quantitations were standardized to amount of total RNA.

**Analysis of microbiota from the mosquito midgut.** DNA extraction and clone library construction were carried out as previously described[51] with the following modifications. Briefly, DNA was extracted from 20 dissected midguts in laboratory-reared mosquitoes using the DNeasy Blood & Tissue kit (Qiagen) and 16S rRNA genes were amplified using primers 8FE and 1490R (Table S4). PCR amplicons were purified with a QIAEX II gel extraction kit (Qiagen) and then used in a second PCR reaction employing primers 8FE and 530R (Table S4). PCR products were then cloned into *E. coli* DH5α using pGEM-T Easy Vectors (Promega) and 107 clones were picked for sequencing. Clones were sequenced and blasted against 16S ribosomal RNA sequences database to verify the species.

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## Author contributions

H.B. and H.K. designed the experiments. H.B. carried out the whole experiments and analyzed the data. B.N. and H.A. contributed to the data analysis and discussion. W.M.G.,

## Additional information

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

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