A specified midgut region controlling the symbiont titer in an insect-microbe gut symbiotic association

Running title: Insect midgut region with antimicrobial activity

Jiyeun Kate Kim¹, Na Hyang Kim¹, Ho Am Jang¹, Yoshitomo Kikuchi², Chan-Hee Kim¹, Takema Fukatsu³#, Bok Luel Lee¹#

Global Research Laboratory of Insect Symbiosis, College of Pharmacy, Pusan National University, Pusan 609-735, South Korea¹; National Institute of Advanced Industrial Science and Technology (AIST), Hokkaido Center, Sapporo 062-8517, Japan²; National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan³

#Address correspondence to Bok Luel Lee, brlee@pusan.ac.kr, or Takema Fukatsu, t-fukatsu@aist.go.jp.

Keywords: gut symbiosis, Riptortus pedestris, Burkholderia, symbiont titer
Many insects possess symbiotic bacteria that affect the biology of the host. The level of the symbiont titer in the host is a pivotal factor that modulates the biological outcome of the symbiotic association. Hence, the symbiont titer should be maintained at a proper level by host’s controlling mechanisms. Several mechanisms for controlling intracellular symbionts of insects have been reported, while mechanisms for controlling extracellular gut symbionts of insects are poorly understood. The bean bug \textit{Riptortus pedestris} harbors a $\beta$-proteobacterial extracellular symbiont of the genus \textit{Burkholderia} in the midgut symbiotic organ designated as M4 region. We found that the M4B region, which is directly connected to the M4 region, also harbors \textit{Burkholderia} symbiont cells, but the symbionts therein are mostly dead. A series of experiments demonstrated that the M4B region exhibits an antimicrobial activity, and the antimicrobial activity is specifically potent to the symbiotic \textit{Burkholderia} but not to the cultured \textit{Burkholderia} and other bacteria. The antimicrobial activity of M4B region was detected in symbiotic host insects, being the highest at the fifth instar, but not in aposymbiotic host insects, which suggests the possibility of symbiont-mediated induction of the antimicrobial activity. This antimicrobial activity was not associated with up-regulation of antimicrobial peptides of host. Based on these results, we propose that M4B region is a specialized gut region of \textit{R. pedestris} that plays a critical role in controlling the population of the \textit{Burkholderia} gut symbiont. Molecular basis of the antimicrobial activity is of great interest and deserves future studies.
INTRODUCTION

Many insects possess symbiotic bacteria within their cells, tissues and guts (1). These symbiotic associations have been established for a long time and known to affect the biology of insects in various ways. Some symbionts play indispensable roles, such as providing essential nutrients (2). Other symbionts play conditionally beneficial roles, such as providing defense against natural enemies and adaptation to specific ecological conditions (3). Others may have parasitic or pathogenic effects on their hosts, causing attenuated host fitness and reproduction aberrations (4).

These symbiotic effects on host’s biology tend to be related to the symbiont titer within the host. The level of the symbiont titer affects the host fitness, the fidelity of vertical transmission, and the intensity of the reproductive aberrations (5-8). Therefore, host insects are expected to develop systems to control and maintain the symbiont titers within an optimal range. Previous studies have reported or suggested several mechanisms for controlling symbiont populations, including mechanisms mediated by lysozymes for controlling Buchnera in aphids (9, 10), reactive oxygen species production for controlling Wolbachia in mosquitos (11), and antimicrobial peptides for controlling Sitophilus primary endosymbionts in weevils (12).

However, these previous studies have mostly focused on the intracellular symbionts of insects, while the mechanisms for controlling the extracellular gut symbionts of insects are poorly understood.

The bean bug Riptortus pedestris harbors a beneficial and specific bacterium of the genus Burkholderia in a specialized region of the posterior midgut (13). This symbiont, which is orally acquired by host nymphs from the environment every generation, is easily cultivable and genetically manipulable (14-16). Hence, the Riptortus-Burkholderia symbiotic system has been
recognized as a promising model to study insect-microbe symbioses in the molecular level.

Using this model system, we report a previously unrecognized insect organ with antimicrobial activity, which is presumably specialized for controlling the gut symbiont population.

**MATERIALS AND METHODS**

**Insect rearing.** The bean bugs *R. pedestris* were reared in our insect laboratory at 28°C under a long day regime of 16 h light and 8 h dark as described (17). The insects were originally collected from fields of the soybean *Glycine max* at Tsukuba, Ibaraki, Japan, from which a laboratory strain TKS-1 has been established (14). Approximately 200 nymphal insects were reared in each of clean plastic containers (34 cm x 19.5 cm wide and 27.5 cm high) supplied with soybean seeds and DWA (distilled water containing 0.05% ascorbic acid). The containers were cleaned every day, and the soybean seeds and DWA were replaced with new ones every two days. Upon reaching adulthood, the insects were transferred to larger containers (35 cm x 35 cm wide and 40 cm high) in which soybean plant pots were provided for food and cotton pads were attached to the walls for egg laying. Eggs were collected daily and transferred to new cages for hatching.

**Burkholderia symbiont inoculation.** *Burkholderia* symbiont strain RPE75, which is a spontaneous rifampicin-resistant mutant derived from the strain RPE64 (15, 16), was cultured at 30°C in YG-RIF medium (YG medium, 0.5% yeast extract, 0.4% glucose, and 0.1% NaCl, containing 30 μg/ml rifampicin). The inoculum solution was prepared by suspending mid-log phase cultured *Burkholderia* cells in DWA at a concentration of $10^7$ cells/ml. Newly molted second instar nymphs were provided with wet cotton balls soaked with the inoculum solution.
After the insects were fed with the inoculum solution for two days, fresh and sterile DWA was provided to the insects instead of the inoculum solution (18).

**Quantitative PCR.** Quantitative PCR for estimating the titers of the *Burkholderia* symbiont was performed as described (17). Dissected midgut samples were subjected to DNA extraction with the QIAamp DNA mini kit (QIAGEN). The DNA samples were mixed with a master PCR solution containing 2 x qPCR premix from the QuantiMix SYBR Kit (PhileKorea) and the primers BSdnaA-F (5’-AGC GCG AGA TCA GAC GGT CGT CGA T-3’) and BSdnaA-R (5’-TCC GGC AAG TCG CGC ACG CA-3’), which target a 0.15-kb region of the dnaA gene of the *Burkholderia* symbiont (15). The dnaA gene encodes the chromosomal replication initiator protein DnaA (protein accession number, BAN21755) and is a single-copy gene in the *Burkholderia* symbiont genome (19). The PCR temperature profile was 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. The threshold cycles (C_T) of the midgut samples were applied to a standard curve generated with standard DNA samples containing known dnaA copy numbers to estimate the bacterial titers. To generate the standard curve of the dnaA gene copies, *Burkholderia* symbiont cells were cultured up to the mid-log phase and serially diluted to obtain the samples containing 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 cells per 100 µl. The cell numbers were verified by a colony forming unit (CFU) assay. Each cell sample was subjected to the same procedures of DNA extraction and PCR analysis as described above.

**CFU assay for symbiont titer.** Dissected midgut samples were collected in 50 µl of PB (10 mM phosphate buffer, pH 7.0) and homogenized with a pestle. The homogenized samples were serially diluted and spread on 1.5% agar plates of YG-RIF medium. After two days of
incubation at 30°C, the colonies on the plates were counted and the numbers of symbiont cells in
the samples were calculated as CFU x dilution factor.

Fluorescence in situ hybridization (FISH). FISH analysis of symbiotic midgut regions
was performed as described (16). Two fluorochrome-labeled probes, Alsym16S (5'-ACA CTC
AAA GCC TGC TGC CAG T-3') and BURK129 (5'-CCA CTA CAG GAC ACG TTC-3’) whose 5’
ends were labeled with AlexaFluor555 (16), were simultaneously used to target different regions
of 16S rRNA of the *Burkholderia* symbiont. The symbiotic midgut regions dissected from third
instar nymphs were fixed with 4% paraformaldehyde in phosphate buffer for 1 h at room
temperature. After the fixation, the samples were treated with 0.1% Triton X-100 for 5 min and
washed with PBS (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, pH 7.5).
The samples were incubated overnight in a hybridization buffer (20 mM Tris-HCl, pH 8.0, 0.9 M
NaCl, 0.01% SDS, 30% formamide) containing 50 nM each of the probes at room temperature.
After washing with PBS, the samples were mounted on glass slides with 30% glycerol and
observed under a fluorescent microscope (AX70, Olympus).

CFU assay for measuring antimicrobial activity. The following bacterial cells were
prepared: symbiotic *Burkholderia* RPE75 freshly isolated from M4 as previously described (18);
mid-log phase *Burkholderia* RPE75 cultured at 30°C in YG-RIF medium; mid-log phase
*Escherichia coli* K12 cultured at 37°C in LB medium (1% tryptone, 0.5% yeast extract, and
0.5% NaCl); and mid-log phase *Staphylococcus aureus* RN4220 cultured at 37°C in LB medium.
These bacterial cells were washed and diluted with PB to 500-1,000 CFUs per 50 µl. M4B whole
lysate was prepared by homogenizing M4B samples dissected from ten fifth instar nymphs in
100 µl PB, and the lysate was further diluted to 1:50 and 1:100 with PB. Each sample consisting of 50 µl of the lysate and 50 µl of the bacteria suspension was incubated at room temperature for 15 min before spreading onto YG-RIF agar plates to count the CFUs.

For comparing the M4B lysates between symbiotic and aposymbiotic insects, the M4B whole lysates were centrifuged at 20,000 x g for 15 min, and the supernatant was subjected to protein quantification by the Bradford assay (Bio-Rad). After serial dilution, 50 µl of the lysates at different concentrations (µg protein/ml) was incubated with 50 µl of the symbiotic *Burkholderia* cell suspension. After 15 min of incubation at room temperature, the samples were spread onto YG-RIF agar plates, cultured for two days, and subjected to colony counting.

To examine the sensitivity to heat, the M4B lysate (0.2 µg/ml in PB) was incubated on ice or at 27°C, 37°C, 45°C, or 65°C for 1 hr, or heated at 100°C for 15 min, prior to the incubation with the symbiotic *Burkholderia* cell suspension. The treated lysates (50 µl each) were incubated with cell suspensions of the symbiotic *Burkholderia* (50 µl each), and after 15 min of incubation at room temperature, the samples were spread onto YG-RIF agar plates, cultured for two days, and subjected to colony counting.

**Reverse transcription quantitative PCR.** The M4B regions were dissected from fifth instar nymphs and subjected to total RNA extraction using RiboEx (GeneAll, South Korea). The RNA samples were reverse transcribed using TOPscript RT DryMIX containing oligo-dT primers (Enzymomics, South Korea) to synthesize the cDNA. The cDNA was subjected to real-time quantitative PCR after being mixed with a TOPreal qPCR 2x PreMIX with SYBR green (Enzymomics, South Korea) and 0.25 µM each of the primers listed in Table 1. The PCR temperature profile was set to 95°C for 10 min followed by 40 cycles of 95°C for 10 sec, 60°C
for 15 sec, and 72°C for 20 sec using the CFX96 real time PCR system (Bio-Rad). The comparative C<sub>T</sub> (ΔΔC<sub>T</sub>) method was used to calculate the relative gene expression levels with elongation factor 1 alpha (EF1α) gene of *R. pedestris* (accession number AB591382) as an endogenous control gene.

**RESULTS AND DISCUSSION**

A particular midgut region contains dead *Burkholderia* symbionts. We measured *Burkholderia* symbiont titers in the symbiotic organs of *R. pedestris*, the midgut fourth section (M4) and the bulbous region prior to M4 (M4B) (Fig. 1A). The insects were infected with the *Burkholderia* symbiont strain RPE75 at early second instar and, when they reached the middle fifth instar, the symbiotic organ samples, either M4 alone, M4B alone or M4B plus M4, were dissected from the insects. The symbiont titers in these samples were measured by quantitative PCR (qPCR) assay and CFU assay (Table 2). Unexpectedly, the qPCR results and CFU results exhibited striking discrepancies. Of particular interest was the difference in the symbiont titers in the M4B alone samples. The qPCR assay detected more than 10<sup>6</sup> dnaA copies per M4B, while the CFU assay detected less than 10 bacterial cells per M4B. These results suggested that most dnaA copies detected by qPCR may be derived from dead symbiont cells. Fluorescence in situ hybridization (FISH) of M4B and M4 corroborated this idea: while M4 exhibited strong fluorescent signals, M4B was scarcely stained, probably because of degradation of symbiont’s 16S rRNA in M4B (Fig. 1B). Another interesting observation was the difference in the symbiont titers between the M4 alone samples and the M4B plus M4 samples. In the CFU assay, the symbiont titers in the M4B plus M4 samples were approximately 1,700-fold less than those in the M4 alone samples, suggesting that M4B has a strong negative effect on the symbiont titer.
(Table 2). On the basis of these results, we hypothesized that M4B has an antimicrobial activity against the *Burkholderia* symbiont.

**The antimicrobial activity of M4B midgut region is specifically potent to the symbiotic *Burkholderia***. To investigate the antimicrobial activity of the M4B midgut region, we applied the whole lysate of the M4B samples to the following bacterial cells: symbiotic *Burkholderia* RPE75, cultured *Burkholderia* RPE75, *E. coli* K12 and *S. aureus* RN4220. As shown in figure 2, the CFUs of the cultured *Burkholderia* and *E. coli* were not affected by treatment with the M4B lysate, whereas the CFUs of *S. aureus* were significantly reduced by treatment with the M4B lysate at relatively high concentrations of 1:10, 1:5 and 1:1 dilutions. Strikingly, the CFUs of the symbiotic *Burkholderia* were significantly reduced by treatment with the M4B lysate even at a low concentration of 1:100 dilution, which was equivalent to a half of an M4B/ml. These results confirmed that M4B has a significant antimicrobial activity, and revealed that the antimicrobial activity exhibits high potency specific to the symbiotic *Burkholderia*.

**The antimicrobial activity of the M4B region is a symbiosis-related feature**. We prepared M4B samples from symbiotic and aposymbiotic fifth instar insects, and measured their antimicrobial activities against the symbiotic *Burkholderia*. The M4B lysates of the symbiotic insects exhibited approximately six-fold higher antimicrobial activities (50% inhibition of cell viability [IC50], 1.7 μg/ml) compared with those of the aposymbiotic insects (IC50, 10.0 μg/ml) (Fig. 3A). This result indicated that the antimicrobial activity against the symbiotic *Burkholderia* is associated with the M4B region of the symbiotic host insect, and suggested the possibility that
the presence of the symbiotic *Burkholderia* may induce the antimicrobial activity against the symbiont in M4B.

We further surveyed the antimicrobial activities of the M4B lysates from the symbiotic and aposymbiotic insects at different developmental stages. At a protein concentration of 5 µg/ml, the M4B lysates of the symbiotic insects exhibited remarkably different levels of the antimicrobial activity against the symbiotic *Burkholderia*: fifth instar > fourth instar > third instar = early adult > late adult (Fig. 3B left). This pattern may reflect the symbiont titers within the host insects: a previous study reported the order of symbiont titers as fifth instar > fourth instar > third instar (15), and the symbiont titers declined during adult aging (J. K. Kim *et al.*, unpublished data). On the other hand, even at a protein concentration as high as 50 µg/ml, the M4B lysates of the aposymbiotic insects consistently exhibited little antimicrobial activity (Fig. 3B right). These results confirmed that the antimicrobial activity against the symbiotic *Burkholderia* is certainly associated with M4B of the symbiotic host insect, and demonstrated that the antimicrobial activity is regulated in a development-dependent manner with the highest activity at the fifth instar stage.

The antimicrobial activity of the M4B region against the symbiont is not associated with expression of antimicrobial peptides. In *R. pedestris*, cDNA sequences of three antimicrobial peptides (AMPs) have been identified: defensin-like peptide (AK416895), thanatin-like peptide (AB842298) and pyrrhocoricin-like peptide (AB842297) (20). To address whether these AMPs are responsible for the antimicrobial activity of the M4B region, expression levels of the AMP genes in the M4B region were compared between the symbiotic insects and the aposymbiotic insects. As shown in figure 4A, the expression levels of defensin-like peptide
gene and pyrrhocoricin-like peptide gene were similar between the symbiotic insects and the aposymbiotic insects, whereas the expression levels of thanatin-like peptide gene in M4B were significantly higher in the symbiotic insects than in the aposymbiotic insects. Meanwhile, when compared between the M4B and M4 regions of the symbiotic insects, the expression levels of the AMP genes were either similar to each other (for defensin-like peptide gene) or rather higher in the M4 region than in the M4B region (for pyrrhocoricin-like peptide gene and thanatin-like peptide gene) (Fig. 4B). These results indicate that these AMPs are not responsible for the antimicrobial activity of the M4B region. When the M4B lysates were treated at different temperatures and subsequently tested for their antimicrobial activities against the symbiotic *Burkholderia*, the activities were significantly reduced by treatments at 37°C and 42°C, and completely lost by treatment at 65°C (Fig. 4C). Since insect AMPs are generally heat resistant (21), these results corroborate the idea that the antimicrobial activity of the M4B region is not associated with AMP expression.

**Conclusion and perspective.** In this study, we identified a previously unrecognized insect organ with significant antimicrobial activity, the M4B midgut region, in *R. pedestris*. Interestingly, the antimicrobial activity of M4B was preferentially detected in the *Burkholderia*-harboring symbiotic insects (Fig. 3), suggesting the possibility of symbiont-mediated induction of antimicrobial activity. Also of interest is that the antimicrobial activity of M4B selectively acts on the symbiotic *Burkholderia* (Fig. 2), highlighting a highly specific aspect of the host-symbiont interactions.

Although speculative, several major biological roles of the symbiont-killing action of M4B, which are not mutually exclusive, are conceivable: (i) regulation of the symbiont population; (ii)
recruitment of symbiont biomass for host growth; and (iii) prevention of colonization of non-symbiotic microbial contaminants. Morphologically, the direct connection of M4B to the main symbiotic region M4 (see Fig. 1 A and B) appears to be suitable for performing these tasks: overgrown *Burkholderia* symbiont cells in M4 may continuously flow back to the neighboring M4B, where the bacterial cells may be killed, digested and absorbed. The highest antimicrobial activity in M4B of the symbiotic fifth instar insects (Fig. 3B left) seems to make sense biologically, because fifth instar nymphs require substantial resources for constructing a thick cuticle, wings and gonads for the subsequent adult molting. Considering that not only symbiotic *Burkholderia* but also *S. aureus* was suppressed by M4B lysate (Fig. 2), M4B may play suppressive role on non-symbiotic microbial contaminants acquired orally through host’s feeding. Future studies will focus on identification of the molecule(s) involved in the symbiotic *Burkholderia*-specific antimicrobial activity in the M4B region of *R. pedestris*. These antimicrobial molecules are sensitive to heat (Fig. 4) and thus unlikely to be antimicrobial peptides. A recent expression sequence tag analysis of symbiotic and non-symbiotic midgut regions of *R. pedestris* identified genes of defense-related proteins, such as lysozymes and cathepsin proteases (20). Our ongoing RNA sequencing analysis using next generation sequencers will provide additional candidate genes, from which the antimicrobial substance(s) of interest may be identified. Alternatively, the antimicrobial activity may be attributable to low-molecular-mass secondary metabolite(s), for which metabolomic approaches are needed. We expect that transcriptomic, proteomic, metabolomic and functional analyses of M4B will uncover pivotal molecular aspects underpinning the regulation of the sophisticated *Riptortus-Burkholderia* gut symbiotic association.
Acknowledgements

This work was supported by the Global Research Laboratory Grant of the National Research Foundation of Korea (grant number 2011-0021535) to T.F. and B.L.L.

Reference


Figure legends

**FIG 1** (A) Midgut morphology of *R. pedestris*. The dissected intestine of a third instar nymph shows the morphologically distinct regions (M1, M2, M3, M4B and M4), of which the M4B and M4 regions are symbiotic organs. The yellow dotted circle indicates the midgut regions subjected to the FISH analysis. (B) FISH analysis of the dissected M4B and M4 regions. The yellow dotted lines indicate the outline of the M4B and M4 regions. The red signal in the fluorescence image indicates 16S rRNA of the *Burkholderia* symbiont.

**FIG 2** The specific antimicrobial activity of M4B against symbiotic *Burkholderia*. Cell suspensions of symbiotic *Burkholderia*, cultured *Burkholderia*, *E. coli* and *S. aureus* were subjected to CFU assays after incubation with M4B lysate samples. PB, phosphate buffer without lysate; M4B WL, whole lysate of M4B midgut region. As for concentration of M4 lysates, M4B WL (1:1) is equivalent to fifty dissected M4Bs/ml, and M4B WL (1:50) is equivalent to a dissected M4B/ml. Means and standard deviations are shown (n = 3). Asterisks indicate statistically significant differences (unpaired t-test: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.005).

**FIG 3** Symbiosis-related antimicrobial activity of M4B. (A) Comparison of the M4B antimicrobial activity between symbiotic (SYM) insects and aposymbiotic (APO) insects. Symbiotic *Burkholderia* isolated from M4 were incubated with different concentrations of SYM M4B lysates or APO M4B lysates, and subjected to CFU assay. Means and standard errors are plotted (n = 3). (B) M4B antimicrobial activities at different development stages. The symbiotic *Burkholderia* were incubated with SYM M4B lysate (left panel) or with APO M4B lysate (right...
panel) prepared from different instar stages. 3rd, third instar; 4th, fourth instar; 5th, fifth instar; A1, early adult (within three days after adult molting); A2, late adult (approximately ten days after adult molting). Means and standard errors are plotted (n = 3 to 6). Different letters (a, b, c) indicate statistically significant differences (unpaired t-test with Bonferroni’s correction: *P < 0.05).

FIG 4 (A and B) Expression levels of the AMP genes in symbiotic organs of fifth instar nymphs of *R. pedestris*. AMP expressions were compared between M4B regions from symbiotic and aposymbiotic insects (A) and between M4B region and M4 region of symbiotic insects (B). To calculate the normalized fold expression \(2^{-\Delta\Delta C_T}\), EF1a was used as a reference gene, and the expression levels were normalized according to the expression of thanatin-like peptide gene in symbiotic insects as 1. Means and standard deviations are shown (n = 3). Asterisks indicate statistically significant differences (unpaired t-test: *, *P < 0.05). (C) Antimicrobial activity test of M4B lysates treated at different temperatures. M4B lysate (0.2 µg/ml in PB) was temperature-treated prior to testing the antimicrobial activity against symbiotic *Burkholderia*. Statistically significant differences between the bacteria CFUs before (PB) and after incubation with M4B lysate are indicated with asterisks. (unpaired t-test: *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001; n = 3).
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defensin-like peptide</td>
<td>TCGGTCCGGACTGAGACTGAA</td>
<td>TTGCCGGCTTTGTATCCCTT</td>
</tr>
<tr>
<td>Pyrrhocoricin-like peptide</td>
<td>TCCGAAGCTGAGGGTCTTCCC</td>
<td>TCCGATCCAAAGTTCCGTCGTC</td>
</tr>
<tr>
<td>Thanatin-like peptide</td>
<td>GTCTGCCTTCGTGAAGACG</td>
<td>ATTCGCTTGCAAACGCCG</td>
</tr>
<tr>
<td>Elongation factor 1 alpha</td>
<td>CCTGCATCCGGTTTTTGT</td>
<td>GGATCGAGGGCTTTCAAATAA</td>
</tr>
</tbody>
</table>
TABLE 2  Symbiont titers in the symbiotic organs, M4 and M4B midgut regions, in *R. pedestris*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Symbiont titer (dnaA copies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4B</td>
<td>$1.88 \times 10^6 \pm 1.18 \times 10^5$</td>
</tr>
<tr>
<td>M4</td>
<td>$2.93 \times 10^8 \pm 8.22 \times 10^7$</td>
</tr>
<tr>
<td>M4 plus M4B</td>
<td>$1.56 \times 10^8 \pm 4.71 \times 10^7$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Symbiont titer (CFUs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4B</td>
<td>6.00 ± 2.00</td>
</tr>
<tr>
<td>M4</td>
<td>$1.26 \times 10^7 \pm 1.70 \times 10^6$</td>
</tr>
<tr>
<td>M4 plus M4B</td>
<td>$7.42 \times 10^7 \pm 1.77 \times 10^6$</td>
</tr>
</tbody>
</table>

*Dissected from fifth instar nymphs of *R. pedestris*.  
*Mean ± standard deviation (n = 3).*