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Supplementary Information for

Weevil *pgrp-lb* prevents endosymbiont TCT dissemination and chronic host systemic immune activation

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This PDF file includes:

Supplementary Material and Methods
Figs. S1 to S7
Tables S1 to S3
Supplementary sequences
References for SI reference citations

Other supplementary materials for this manuscript include the following:

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Supplementary Information Text

Supplementary Material and Methods

Multiple sequence alignments

Multiple sequence alignments were performed using the Clustal Omega program [1]. Uniprot accession numbers are as follows: *Sodalis pierantonius* MurE: W0HJN2; *Escherichia coli* MurE: P22188; *Bacillus subtilis* MurE: Q03523; *Helicobacter pylori* MurE: E1S713; *Pseudomonas aeruginosa* MurE: A0A0F6UH44; *Vibrio cholera* MurE: Q9X6N4; *Staphylococcus aureus*: Q2FZP6 MurE; *Streptococcus pneumonia* MurE: A0A0E8T7T8; *Enterococcus faecalis* MurE: Q838A4; *Glossina morsitans* PGRP-LB: A0A1B0G8B0; *Drosophila melanogaster* PGRP-LB: Q8INK6; *Euprymna scolopes* PGRP2: Q32S45; *Drosophila melanogaster* PGRP-LCx: Q9GNK5-2; *Drosophila melanogaster* PGRP-SA: Q9VYX7; *Homo sapiens* PGLYRP1: O75594; *Homo sapiens* PGLYRP3: Q96LB9.

***S. pierantonius* PG analysis**

300 bacteriomes from *S. zeamais* fourth instar larvae were dissected. This material was resuspended into 500 µl of 4.5% SDS solution at 95-100°C and extraction was continued for one hour at this temperature under agitation. PG-containing pellets were recovered by centrifugation at 200,000 *g* for 30 min at room temperature with a Beckman TL-100 centrifuge and they were subsequently washed several times with water. The final pellets were resuspended in 400 µL of 6 M HCl and hydrolysis was performed for 16 h at 96°C. The amino acid and hexosamine composition of hydrolysates was then analyzed with a Hitachi L8800 amino acid analyzer (ScienceTec).

Sample Preparation for Histology

Samples were fixed in PFA 4%. After one week at 4°C, the fixative was replaced by several washings with PBS before embedding the tissue in 1.3% agar. Subsequently, samples were dehydrated through a graded ethanol (EtOH) series and transferred to butanol-1, at 4°C, overnight. Samples in agar were then embedded in melted Paraplast. Tissue sections (3 µm thick) were cut using an HM 340 E electronic rotary microtome (ThermoFisher Scientific). Sections were placed on poly-lysine-coated slides, dried overnight in a 37°C oven, and stored at 4°C prior to further treatments.

PGRP-LB Immunostaining

Paraffin sections were dewaxed twice in methylcyclohexane for 10 min, rinsed in EtOH 100°, and rehydrated through an ethanol gradient to PBS. Slides were incubated with 1% Bovine Serum Albumine (BSA) in PBS for 30 min prior to primary antibody incubation, overnight at 4°C. Rabbit polyclonal anti-serum directed against PGRP-LB (anti-PGRP-LB antibody, used in Vallier et al., 2009) was used as the primary antibody. Preimmune rabbit serum (J0) was used as a negative control. Antibody specificity was checked by western blot [2] and immunostainings

(Figure S7B) on RNAi pgrp-lb individuals. All antisera were diluted at 1:500 in PBS containing 0.1% BSA. After primary antiserum incubation, sections were washed with PBS containing 0.2% Tween. Primary antibodies were detected with fluorescent donkey anti-rabbit IgG, labeled with Alexa Fluor 488. This secondary antibody was applied for 1 h at room temperature, diluted at 1:600 in 0.1% BSA in PBS. They were washed with PBS-Tween, rinsed with PBS and washed several times with tap water. Sections were then dried and mounted using PermaFluorTM Aqueous Mounting Medium (ThermoFisher Scientific), together with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for nuclear staining (3 μg/ml of medium). Images were acquired using an epifluorescence microscope (Olympus IX81), under specific emission filters: HQ535/50 for the green signal (antibody staining), D470/40 for the blue signal (DAPI) and HQ610/75 for the red signal (unspecific autofluorescence from tissue). Images were captured using an XM10 camera and the CellSens Software (Soft Imaging System). Images were treated using ImageJ (release 1.47v). For image treatment, each color channel was treated independently.

RNA isolation and libraries construction for RNA-seq

For *S. oryzae*, RNA-seq datasets were retrieved from the Sequence Read Archive (SRX1034967-SRX1034972 and SRX3721133-SRX3721138). RNA-seq libraries for two other species, *S. zeamais* and *S. linearis* were constructed from a mix of two different pools of adult weevils, naïve and challenged with Gram-positive (*M. luteus*, strain CCM169) or Gram-negative (*D. dadantii,* strain A470) bacteria. Five individuals were randomly sampled at 6 h and 12 h post-injection for each bacterial challenge leading to a total of 20 adults per weevil species. Total RNA from these 20 adults was extracted with Trizol reagent (ThermoFisher Scientific) following the manufacturer's instructions. RNA was incubated with 1 U/μg of RQ1 RNase-free DNase (Promega) for 30 min, at 37°C then purified with the NucleoSpin RNA Clean-up kit (Macherey-Nagel). Total RNA concentration and quality were checked using the Qubit Fluorometer (ThermoFisher Scientific) and the Agilent 2200 TapeStation (Agilent Technologies). 20 naïve adults were randomly selected for *S. zeamais* and *S. linearis* weevil species and total RNA was extracted using the same procedure. Two different pools were prepared for the two weevil species respectively, by mixing 1 μg of RNA from naïve weevils with 1 μg of RNA from weevils challenged with bacteria. RNA-seq libraries were then prepared using the SENSE mRNA-seq library preparation kit (Lexogen) following the manufacturer's instructions and sequenced on an Illumina NextSeq500 platform (2x150bp, Illumina) at the sequencing platform of the IGFL (Institut de Génomique Fonctionnelle de Lyon, Ecole Normale Supérieure de Lyon, France).

Transcriptome assembly and identification of PGRP-LB transcripts

The raw reads were first processed using cutadapt v1.16 [3] to remove adapters and trim low-quality ends ($Q < 20$). Transcriptome assembly was performed using Trinity v2.3.2 [4] for each weevil species. *pgrp-lb* transcripts were identified using TBLASTN [5] with the *D. melanogaster* PGRP-LB protein sequence (Uniprot: Q8INK6) as query. All data from this study have been deposited at the NCBI Sequence Read Archive (SRA) under the following accession numbers: *S. zeamais*: SRP149413; *S. linearis*: SRP149424.

PCR amplification of *pgrp-lb* transcripts

PCR amplification of *pgrp-lb* transcripts was performed with a Taq'ozyme kit (Ozyme) on total cDNA from *S. zeamais* (bacteriome extracts for *pgrp-lbi* and *pgrp-lbt*, challenged whole larvae for *pgrp-lbe*). Primers used are listed in **Table S3**. 1% agarose gel electrophoresis was then performed in order to visualize the amplified fragments.

PGRP-LBi production and purification

PGRP-LBi was expressed, produced and purified in *E. coli* as previously described [2], and used for antibody production, biochemical assays, and bactericidal activity. The purified protein was concentrated and buffer-exchanged in Amicon Ultra 10K centrifugal filters from Merck Millipore:

- to buffer containing 0.05 M Tris-HCl (pH 8.8), 1 mM EDTA and 10% glycerol for antibody preparation.
- to buffer containing 0.02 M HEPES pH 7.4, 2 mM ZnSO4, 4 mM 2-mercaptoethanol and 10% glycerol for enzymatic activity assay and bactericidal activity assay.

DNA constructions for *pgrp-lb* expression in S2 *Drosophila* cells

pgrp-lbi was amplified from *S. zeamais* cDNA by PCR using the primers pgrp-lbi-S2-EcoRI-for and pgrp-lbi-S2-EcoRV-rev, containing respectively EcoRI and EcoRV restriction sites. The PCR product was then inserted into the EcoRI and EcoRV sites of a pMT-V5-HisA plasmid (ThermoFisher Scientific) to produce pMT_*pgrp-lbi*_V5-His. This construct was used to produce a V5 C-terminally tagged PGRP-LBi (Construction (i), **Figure S3A**).

pgrp-lbt was amplified from *S. zeamais* cDNA by PCR using the primers pgrp-lbt-S2-EcoRV-for and pgrp-lbt-S2-XhoI-rev, containing respectively EcoRV and XhoI restriction sites. The PCR product was then inserted into the EcoRV and XhoI sites of a pMT-V5-HisA plasmid (ThermoFisher Scientific) to produce pMT_*pgrp-lbt*_V5-His. This construct was used to produce a C-terminally V5 tagged PGRP-LBt (Construction (ii), **Figure S3A**).

The *egfp* gene coding sequence was amplified from pEGFP-C1 (Clontech) by PCR using the primers Fw_EGFP_KpnI and Rv_EGFP_EcoV and the product was inserted into the KpnI and EcoRV site of plasmid pMT_*pgrp-lbt*_V5-His to produce pEGFP_*pgrp-lbt*_V5-His. This construct was used to produce an N-terminally EGFP and V5 C-terminally tagged PGRP-LBt (Construction (iii), **Figure S3A**).

All constructs were verified by DNA sequencing, and constructions were then used for ectopic expression in S2 *Drosophila* cells. All the primers used are listed in **Table S3**.

***pgrp-lb* expression in S2 *Drosophila* cells and immunostainings**

S2 cells were thawed and put in culture in Schneider medium (Invitrogen) containing fetal bovine serum (FBS, Fisher Scientific) 10% and penicillin-streptomycin 1% (Euromedex). 8-well Nunc® Lab-Tek® Chamber Slide™ systems (Sigma-Aldrich) were filled with 500 µL of S2 cells at 10^6 cells/mL. Cell transfection was performed by adding in each well: 100µL Schneider medium, 4 µL transfectin, 100 ng of construction (i), (ii) or (iii), and 350 µL Schneider medium containing FBS 10% and penicillin-streptomycin 1%. The day after, plasmid expression was induced by adding 2.5 µL of $CuSO_4$ 100mM in each well. Immunostainings were performed two days later. Cells were fixed with 500 µL PFA 4% during 10 min, washed repeatedly with PBS, permeabilized with PBS + triton 0.2% for 5 min. After washing with PBS, wells were blocked

with 500 µL PBS + goat serum 5% (Sigma-Aldrich) for 1h, and rinsed with PBS. Wells were incubated with primary antibodies for 3 hours at room temperature (mouse anti-V5 antibody diluted 1:500, or rabbit anti-EGFP antibody diluted 1:200, Fisher Scientific). Wells were washed with PBS and incubated for 2 h at room temperature with secondary antibodies (Alexa Fluor® 647 goat anti-mouse IgG diluted 1:5000, ThermoFisher Scientific, or Rhodamine Red™-X goat anti-rabbit IgG, diluted 1:500, Molecular Probes). Wells were washed with PBS, water, and removed from the slide. Slides were dried, and mounted using PermaFluor™ Aqueous Mounting Medium (ThermoFisher Scientific), together with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for nuclear staining (3 µg/ml of medium). Slides were observed with a LSM800 confocal microscope (Zeiss) and images were taken with the Zen software.

DNA construction for *pgrp-lbt*-specific dsRNA synthesis

The size of the portion encoding the transmembrane domain (hereafter referred to as 'TM-domain'), specific to *pgrp-lbt*, is too small (115 bp) to generate a functional dsRNA in order to specifically inhibit *pgrp-lbt* transcripts. To overcome this problem, we generated a repetitive fragment of the cDNA coding for the TM-domain, long enough to trigger the RNAi machinery (194 bp), all the while preserving unique 5' and 3' extremities for PCR amplification with primers 5-pgrp-lbt-T7 and 3-pgrp-lbt-T7. The procedure is illustrated in **Figure S7C**.

First, TM-domain-fragment1 **(Figure S7C)** was amplified from *S. zeamais* total cDNA by PCR with 5_TM-domain_F1 and 3_TM-domain_F1_ClaI primers. The fragment 1 (99 bp) was cloned into a pCR2.1-Topo expression vector (Invitrogen). Then, TM-domain-fragment2 (104 bp, **Figure S7C**) was amplified from *S. zeamais* total cDNA by PCR with 5_TM-domain_F2_ClaI and 3_TM-domain_F2_ClaI primers with ClaI SR at each extremities. Finally, pCR2.1-topo-TM-domain-fragment1 and PCR product TM-domain-fragment2 were digested by ClaI, ligated and transformed into an *E. coli* TOP10 strain. The final construction (pCR2.1-topo-TM-domain-fragment1-fragment2) was used as a template for dsRNA synthesis. All constructs were verified by DNA sequencing. All primers used are listed in **Table S3**.

DNA Extraction and qPCR Bacterial Count

DNA extraction and symbiont density quantification was performed as previously described [6].

***S. pierantonius* localization by Fluorescence *in situ* Hybridization**

After methylcyclohexan dewaxing, deproteinization of slides was performed in hydrochlorid acid 0.01 N with pepsin 0.1 mg/mL for 10 min at 37 °C. The sections were then prehybridized, hybridized with a *S. pierantonius* specific 5′-end TAMRA-labeled oligo-probe targeting 16S RNA (TAMRA-ACC-CCC-CTC-TAC-GAG-AC-3',10 µg/mL), washed and then mounted in PermaFluor Mounting Fluid (ThermoFisher Scientific) containing 3 µg/mL of 4′,6-diamidino-2-phenylindole (DAPI), as previously described [7]. Images were acquired the same way as described in the "PGRP-LB immunostaining" part.

PGRP-LBi bactericidal assays

E. coli (strain MG1655) was grown in LB medium to DO_{600}=0.6. In each well of a 96-well plate, 70 µL of bacterial culture were added, as well as 10 µL ethanolamine 0.5 M pH 9.6, 1.25 µL of sterile water or $ZnSO_4$ 200 mM, and 20 µL of HEPES 20 mM pH 7.4, 2-mercapto-ethanol 4 mM,

glycerol 10% containing PGRP-LBi at concentrations from 0 to 500 µg/mL. A well with sterile LB medium instead of bacteria was included to measure the background signal, and a well containing ampicillin was included as a positive control of a bactericidal activity. Each well was repeated twice in the same plate. The plate was incubated for 18 h at 30°C and DO_{600} was then measured in each well with a PowerWave XS plate reader (BioTek).

Armadillo and Cut immunostainings

Armadillo and Cut localization were performed with the same protocol as the PGRP-LB immunostainings. The anti-Armadillo antibody, developed by E. Wieschaus, and the anti-Cut antibody, developed by G. M. Rubin, were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. The anti-Armadillo antibody was used at a 1:4 dilution, and the anti-Cut at a 1:10 dilution, as previously described [8]. An Alexa Fluor® 647 goat anti-mouse IgG diluted 1:500 (ThermoFisher Scientific) was used as the secondary antibody in both cases. Images were acquired the same way as described in the "PGRP-LB immunostaining" part.

Egg count in female ovaries

Following dsRNA injection at day 1 after final ecdysis, female ovaries were dissected from day 4 to day 8, and at day 15 after ecdysis in diethylpyrocarbonate-treated Buffer A (25 mM KCl, 10 mM $MgCl_2$, 250 mM Sucrose, 35 mM Tris/HCl, pH=7.5). Eggs were counted in five ovaries by condition.

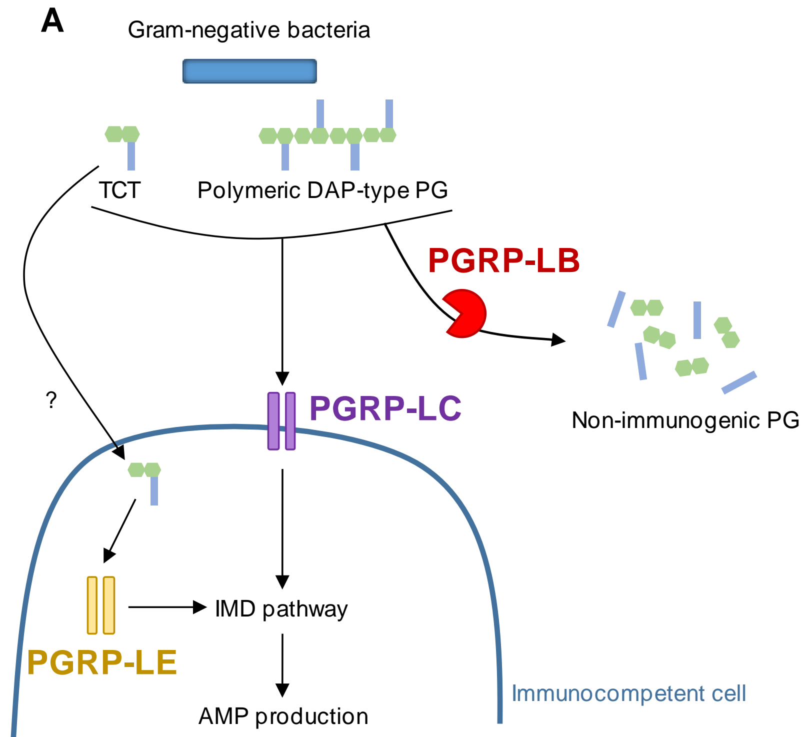
Identification of genes of interest and accession numbers

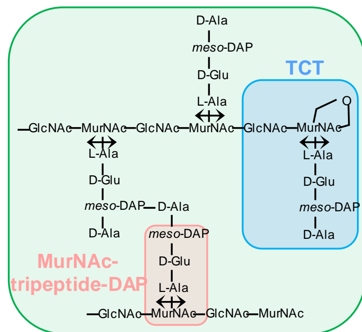
Genes encoding *colA, colB, sarcotoxin, imd,* and *relish* in *S. zeamais* were identified in previous studies [9–11]. *pgrp-lc* was first identified in a previously published *S. oryzae* transcriptome [12], based on the presence of a PG recognition domain, the absence of enzymatic activity, the presence of a RHIM domain and a transmembrane domain. A partial sequence was then recovered in *S. zeamais* based on sequence similarity with *S. oryzae*, and was subsequently amplified and sequenced (accession number: MH423625). *pgrp-lb* transcript sequences were identified from the transcriptomic data published in this study (accession number SRP149413), and were subsequently amplified and sequenced in *S. zeamais*. Accession number are as follows: *pgrp-lbi*: MH423627, *pgrp-lbt*: MH423628, *pgrp-lbe*: MH423629.

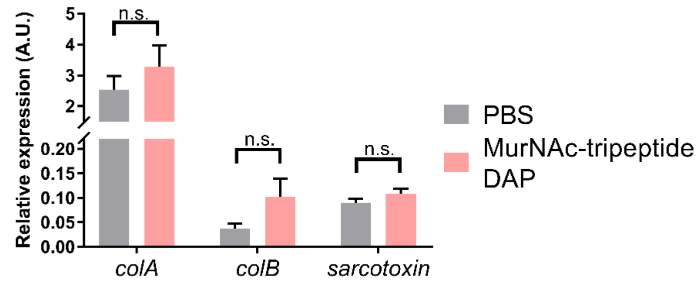
Statistical analysis

Transcriptomic data on immune response kinetics **(Figure 2A)** were analyzed by fitting a Generalized Linear Model (GLM) with a Gamma transformation to the data. The type of PG injected was considered as a 3-level categorical variable representing the injection of sterile PBS (control), TCT, or polymeric DAP-type PG. The time post-infection was considered as a 4-level categorical variable. The impact of TCT or polymeric DAP-type PG was assessed by comparing with a t-test the regression coefficients specific to those factors, with those of PBS injection results. Other transcriptomic data were analyzed doing pairwise comparisons using a Welch t-test on the log-transformed gene expression data. Symbiont quantification data and egg load data were analyzed doing pairwise comparisons using a Student t-test.

All analyses and graphical figures were made using RStudio software v0.98.983 and Hmisc and nlme packages, as well as GraphPad Prism v7 for Windows. The effect of a factor was considered to be significant with a p-value <0.05. Graphical figures represent the mean of all replicates for each point. Error bars represent the standard error calculated as $\sigma / \sqrt{n}$, where σ is the standard deviation and n is the number of replicates.


A
Gram-negative bacteria
TCT
Polymeric DAP-type PG
PGRP-LB
Non-immunogenic PG
?
PGRP-LC
IMD pathway
PGRP-LE
AMP production
Immunocompetent cell


B
Polymeric DAP-type PG
TCT
MurNAc-tripeptide-DAP


C
Relative expression (A.U.)
n.s.
colA
colB
sarcotoxin
PBS
MurNAc-tripeptide DAP

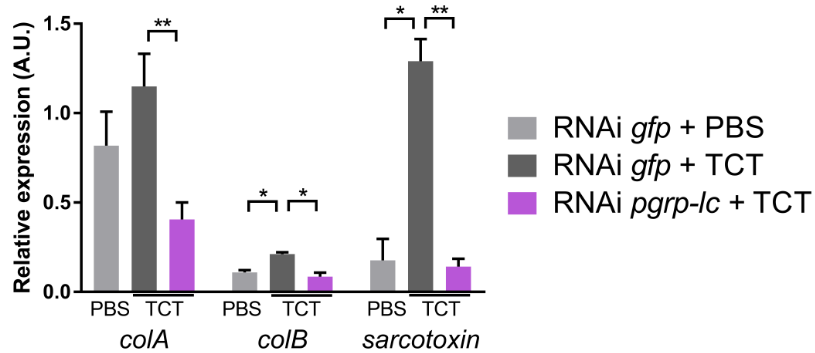

D
Bacteriomes
Relative expression (A.U.)
PBS
TCT
colA
colB
sarcotoxin
RNAi gfp + PBS
RNAi gfp + TCT
RNAi pgrp-lc + TCT

Figure S1: **Peptidoglycan recognition and structure**. **Related to Figure 2. A:** Schematic representation of PGRP-LC, -LE, and -LB function in the IMD pathway activation and regulation by bacterial PG in *Drosophila* [13]. DAP-type PG from Gram-negative bacteria can be monomeric (TCT) or polymeric (see panel B). The transmembrane receptor PGRP-LC recognizes both monomeric and polymeric circulating DAP-type PG. The intracellular isoform of the receptor PGRP-LE can only recognize TCT when located within insect cells. Both receptors lead to the IMD pathway activation, which upregulates AMP-encoding gene expression. PGRP-LB is an amidase that cleaves DAP-type PG into non-immunogenic fragments, therefore inhibiting the IMD pathway. **B:** Structure of DAP-type PG and monomeric fragments used in this study. Chains of DAP-type PG end with a 1,6-anhydro-MurNAc; when released, the terminal disaccharide-tetrapeptide motif corresponds to TCT (in green). Arrows indicate *N*-acetylmuramoyl-L-alanine amidase cleavage sites. **C:** Systemic AMP-encoding gene expression following MurNac-tripeptide-DAP injection. *colA, colB* and *sarcotoxin* expression were measured in carcasses by qRT-PCR six hours after either PBS or *E. coli* MurNAc-tripeptide-DAP injections. A.U.: Arbitrary Units. The mean and standard error for five independent replicates are represented. 'n.s.' indicates a non-significant difference between two conditions based on a Welch's t-test. **D:** AMP-encoding gene expression in the bacteriome following *pgrp-lc* inhibition by RNAi. *colA, colB* and *sarcotoxin* expression was measured in bacteriomes by qRT-PCR 6 h after either PBS or TCT injection, six days following *gfp* or *pgrp-lc* dsRNA injection. Local AMP expression does not increase following TCT injection when *pgrp-lc* is inhibited. Asterisks indicate a significant difference between two conditions based on a Welch's t-test. The mean and standard error for five independent replicates are represented. A.U. = Arbitrary Units. n.s.: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

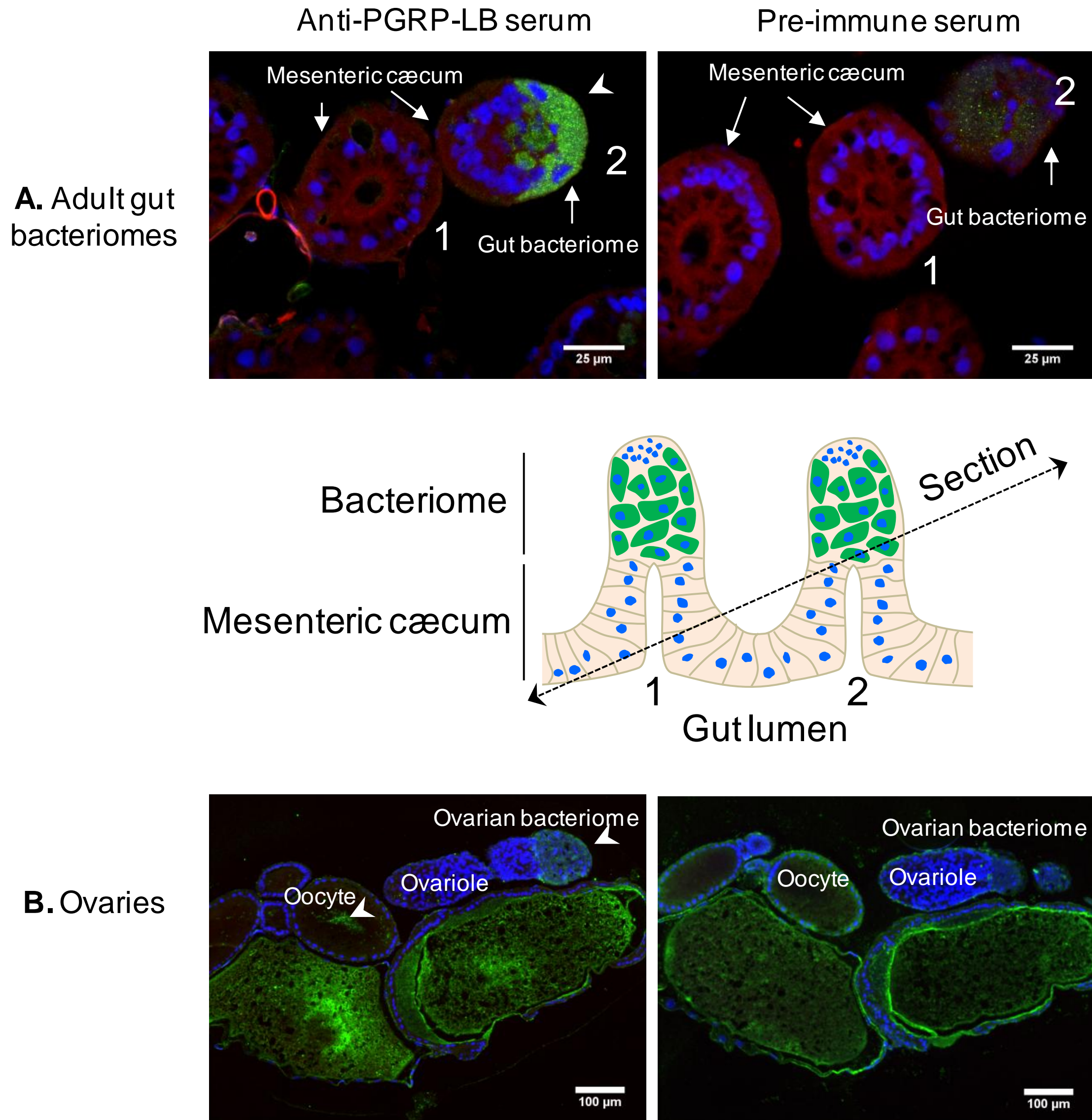


Figure S2: PGRP-LB localization in other tissues by immunostaining. Related to Figure 3. A pre-immune serum was used as the negative control (right column). PGRP-LB-associated signal (white arrowheads) is strongly observed in adult gut bacteriomes **(A)**, oocytes and ovarian bacteriomes **(B)**. Blue: DAPI; green: PGRP-LB; red: autofluorescence. A scheme showing the adult bacteriome morphology is included (bacteriocytes are in green), highlighting how the section was likely performed and the two caeca (1 and 2) observed in the fluorescence microscopy pictures.

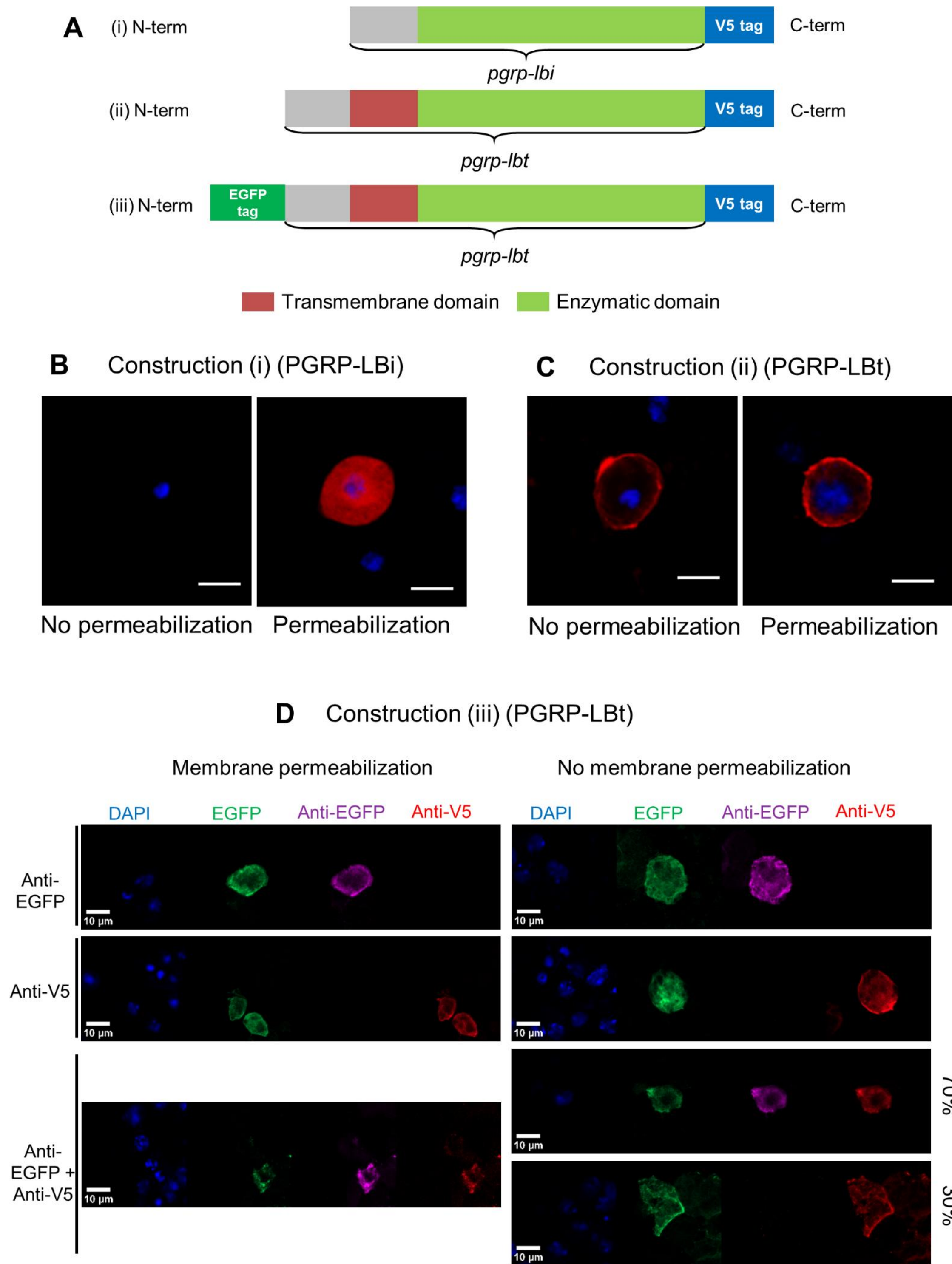
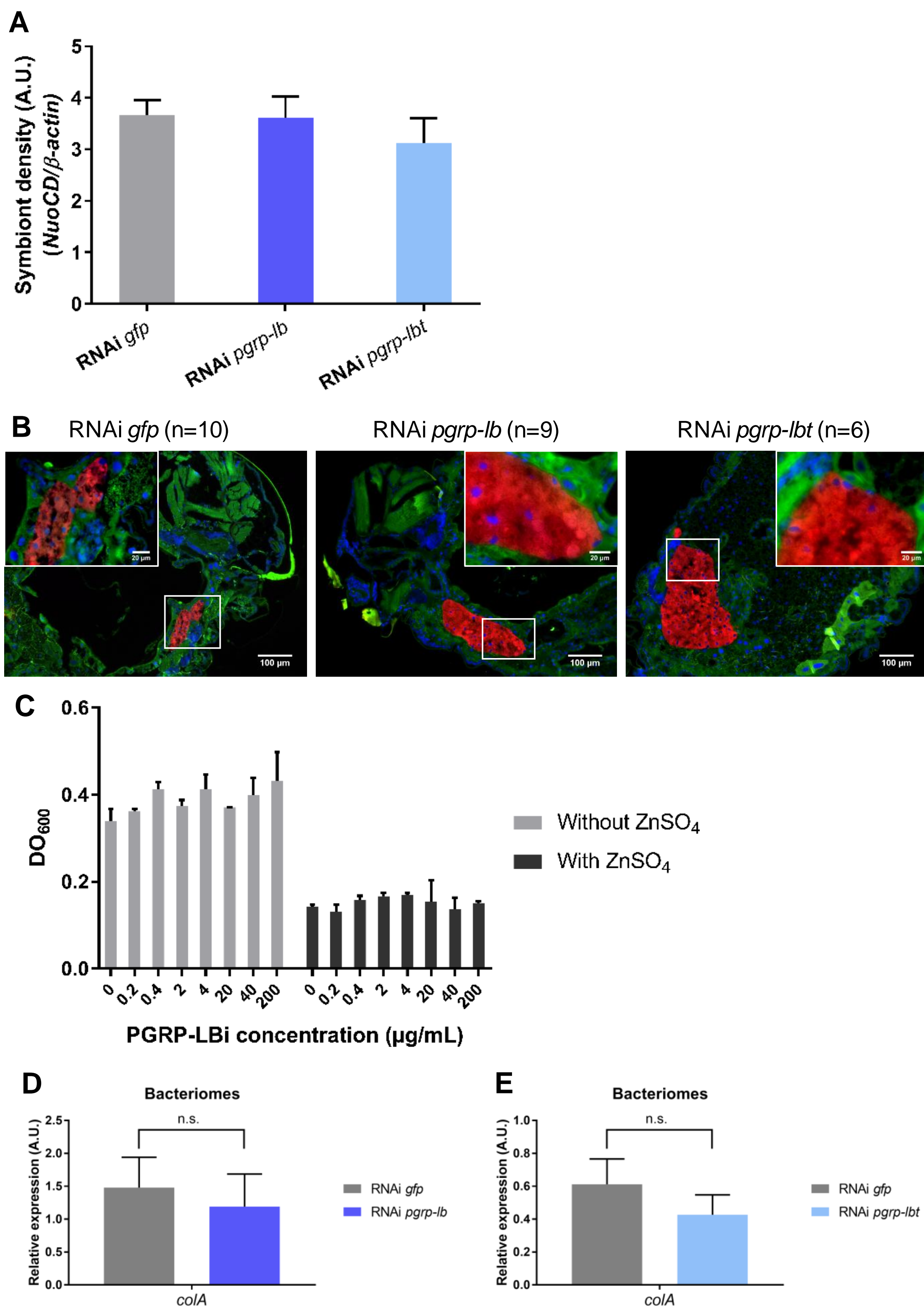


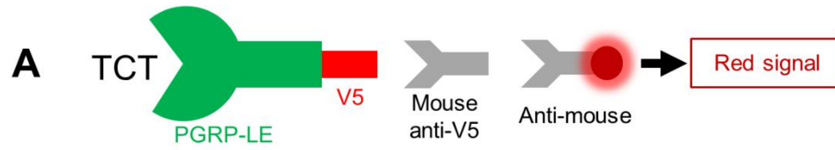
Figure S3: Subcellular localization of PGRP-LBi and PGRP-LBt. Related to Figure 3. A: Constructions (i), (ii), and (iii) were transfected and expressed in S2 *Drosophila* cells. **B-C:** Anti-V5 immunostaining, with (right column) or without (left column) membrane permeabilization and observed by confocal microscopy reveals that PGRP-LBi (B) is associated with intracellular signal while PGRP-LBt (C) is associated with transmembrane signal. Scale bar: 6µm. Blue:

DAPI; red: anti-V5 staining. **D:** Anti-V5 and/or anti-EGFP immunostainings, with (left column) or without (right column) membrane permeabilization, and observed by confocal microscopy reveal PGRP-LBt can adopt both orientations in *Drosophila* membranes. GFP signal was used as a transfection control. Immunostainings performed with (left column) or without (right column) cell permeabilisation all exhibited signal associated with antibodies that were used, *i.e.* magenta signal for anti-EGFP staining, red signal for anti-V5 staining, or both. The latter was only observed in 70% of the cells: the remaining 30% only exhibited anti-V5 signal. Blue: DAPI; green: EGFP; magenta: anti-EGFP staining; red: anti-V5 staining.



A
Symbiont density (A.U.)
(NuoCD/β-actin)
RNAi gfp
RNAi pgrp-lb
RNAi pgrp-lbt
B
RNAi gfp (n=10)
RNAi pgrp-lb (n=9)
RNAi pgrp-lbt (n=6)
20 µm
100 µm
C
DO600
Without ZnSO4
With ZnSO4
PGRP-LBi concentration (µg/mL)
D
Bacteriomes
n.s.
Relative expression (A.U.)
RNAi gfp
RNAi pgrp-lb
colA
E
Bacteriomes
n.s.
Relative expression (A.U.)
RNAi gfp
RNAi pgrp-lbt
colA

Figure S4: *pgrp-lb* inhibition effect on symbiont control and localization. **Related to Figure 7. A:** Global symbiont density in larvae eight days following *pgrp-lb* or *pgrp-lbt* RNAi treatment. Symbiont density was calculated as the ratio between the numbers of *NuoCD* genomic copies (*S. pierantonius* gene) and of *β-actin* genomic copies (*S. zeamais* gene). A.U.: Arbitrary Units. The mean and standard error for eight independent replicates are represented. No significant difference was found based on a Student t-test. **B:** *S. pierantonius* localization by FISH eight days after *pgrp-lb* or *pgrp-lbt* RNAi treatment. No morphological differences were observed and no endosymbiont outside of the bacteriome was seen. Blue: DAPI; green: autofluorescence; red: *S. pierantonius*. **C:** Bactericidal assay of recombinant PGRP-LBi. *E. coli* were incubated for 18 h with increasing concentrations of recombinant PGRP-LBi, with and without $ZnSO_4$ (a necessary compound for amidase activity). The presence of PGRP-LBi had no effect on bacterial growth. **D-E:** *colA* expression in the bacteriome eight days after *pgrp-lb* (D) or *pgrp-lbt* (E) RNAi treatment. *colA* expression was measured in dissected bacteriomes by qRT-PCR eight days after *pgrp-lb* or *pgrp-lbt* extinction. A.U.: Arbitrary Units. The mean and standard error for five independent replicates are represented. No significant difference (n.s.) was found between two conditions based on a Welch's t-test.



B

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MGSHHHHHHSGGKPIPNPLLGLDSTGGELSAIIPRSSWLAQKPMDEPLPL
QLPVKYVVILHTATESSEKRAINVRLIRDMQCFHIESRGWNDIAYNFLVG
CDGNIYEGRGWKTVGAHTLGYNRISLGISFIGCFMKELPTADALNMCRNL
LARGVEDGHISTDYRLICHCQCNSTESPGRRLYEEIQTWPHFYNIEEEEQ
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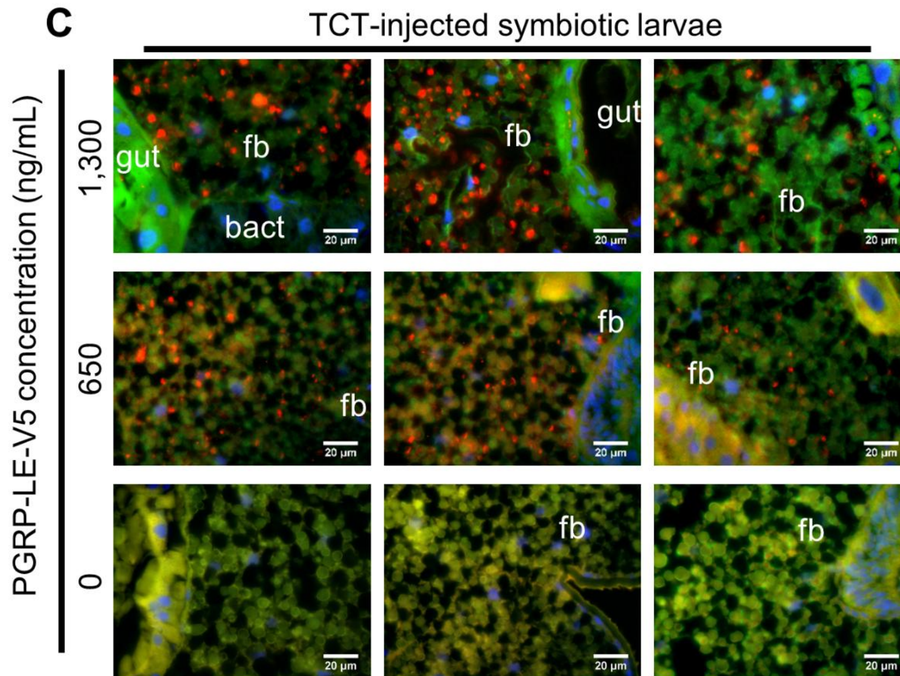
6His-tag with linkers : [1 : 11]
V5-tag : [12 : 25]
PGRP-LE[173-345] : [28 : 200]



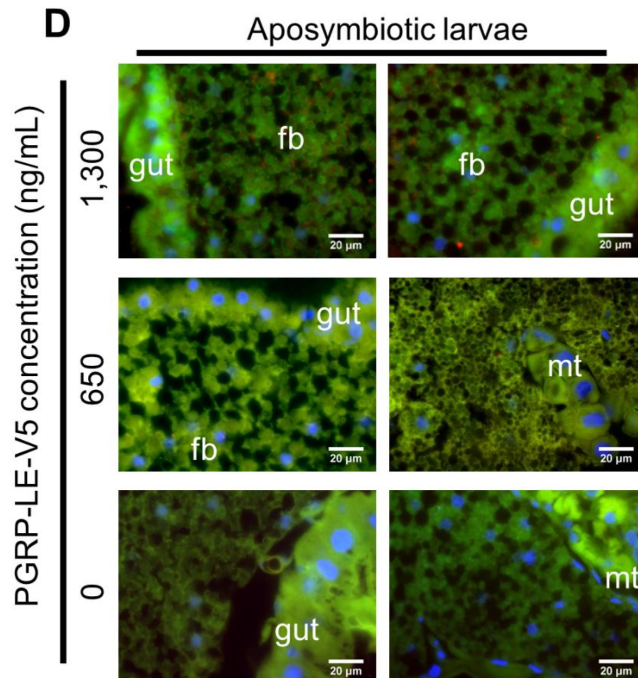


Figure S5: Development and validation of a novel TCT localization tool. Related to Figure 7. A: Schematic representation of the construction and protocol. *Drosophila* PGRP-LE recognition domain is used as a TCT detector, linked to a V5 tag. An anti-V5 immunostaining is then performed to reveal TCT presence. **B:** Protein sequence of the PGRP-LE-V5 construction. Blue: 6His-tag for protein purification. Red: V5-Tag. Green: *Drosophila* PGRP-LE recognition domain. **C:** TCT localization in TCT-injected symbiotic larvae. Larvae were recovered 1 h after the injection in the fat body of 55 nL of a 0.2 mM solution of purified TCT. Two dilutions of PGRP-LE-V5 were tested, as well as a negative control without PGRP-LE (lower panels). Red signal is observed throughout the fat body and is proportional to PGRP-LE-V5 concentration, confirming the efficiency of the method. **D:** TCT localization in naive aposymbiotic larvae, which do not possess any natural source of TCT. Two dilutions of PGRP-LE-V5 were tested in order to evaluate the background signal due to aspecific staining, as well as a negative control without PGRP-LE (lower panels). Faint signal is observed at the higher PGRP-LE-V5 concentration (upper panels), while no signal is observed at the lower dilution (middle panel). The second dilution (650 ng/mL) was thus chosen for any further experiment, since it allows TCT recognition in positive controls (Figure S5C). mt: Malpighian tubules; bact: bacteriome; fb: fat body. Blue: DAPI; green: autofluorescence; red: TCT.

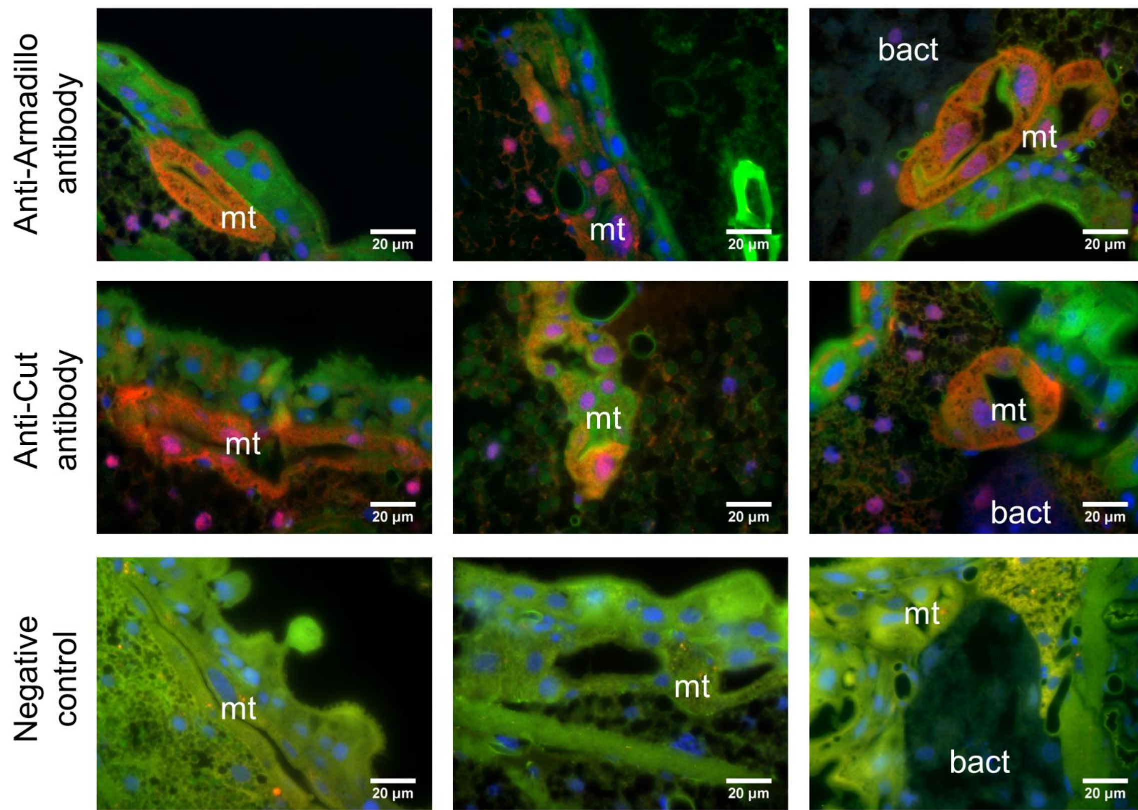


Figure S6: Armadillo (upper panels) and Cut (middle panels) localization in larval tissues by immunostainings. Related to Figure 7. A PBS negative control was included (lower panels). Both proteins are known to be strongly produced in the Malpighian tubules, supporting that the structures in which TCT was previously observed are indeed Malpighian tubules. Blue: DAPI; green: autofluorescence; red: Armadillo (upper panels) or Cut (middle panels).

A

imd

Relative expression (A.U.)

0.20
0.15
0.10
0.05
0.00

***
**

Bacteriomes Carcasses

RNAi *gfp*
RNAi *imd*

relish

Relative expression (A.U.)

0.020
0.015
0.010
0.005
0.000

***
***

Bacteriomes Carcasses

RNAi *gfp*
RNAi *relish*

pgrp-lc

Relative expression (A.U.)

0.010
0.008
0.006
0.004
0.002
0.000

***
***

Bacteriomes Carcasses

RNAi *gfp*
RNAi *pgrp-lc*

Symbiotic adults

Relative expression (A.U.)

0.07
0.06
0.02
0.01
0.0002
0.0001
0.0000

Guts Carc Guts Carc Guts Carc

pgrp-lbi *pgrp-lbt* *pgrp-lbe*

RNAi *gfp*
RNAi *pgrp-lb*
RNAi *pgrp-lbt*

Symbiotic larvae

Relative expression (A.U.)

0.6
0.4
0.2
0.03
0.02
0.01
0.0005
0.0000

Bact Carc Bact Carc Bact Carc

pgrp-lbi *pgrp-lbt* *pgrp-lbe*

RNAi *gfp*
RNAi *pgrp-lb*

Symbiotic larvae

Relative expression (A.U.)

0.2
0.1
0.006
0.004
0.002
0.0004
0.0002
0.0000

Bact Carc Bact Carc Bact Carc

pgrp-lbi *pgrp-lbt* *pgrp-lbe*

RNAi *gfp*
RNAi *pgrp-lbt*

Aposymbiotic larvae

Relative expression (A.U.)

0.015
0.010
0.005
0.000

pgrp-lbi *pgrp-lbt* *pgrp-lbe*

RNAi *gfp*
RNAi *pgrp-lb*

Aposymbiotic larvae

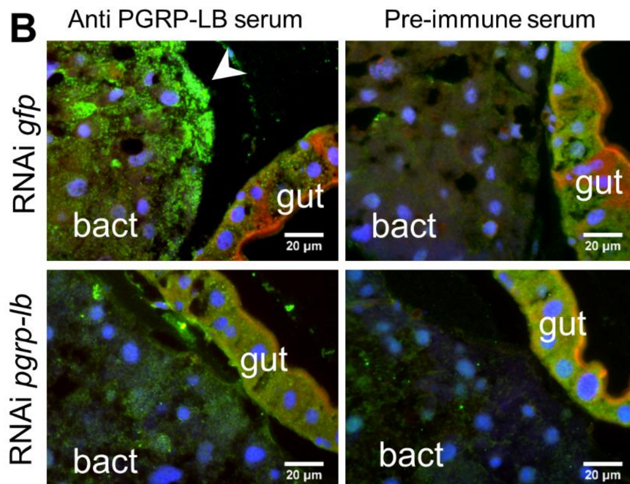
Relative expression (A.U.)

0.015
0.010
0.005
0.000

n.s.
*
n.s.

pgrp-lbi *pgrp-lbt* *pgrp-lbe*

RNAi *gfp*
RNAi *pgrp-lbt*



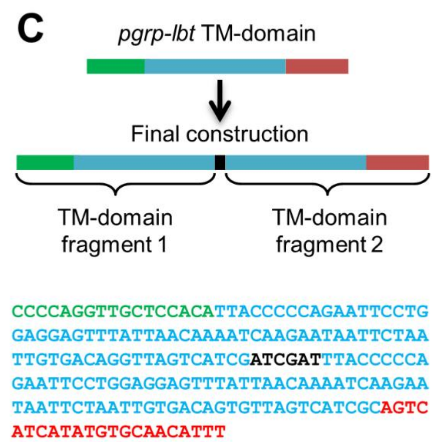


Figure S7: Efficiency of RNAi treatments. Related to the Material and Methods. A: Transcript quantification after its inhibition by RNAi was measured in bacteriomes, carcasses, or aposymbiotic larvae by qRT-PCR six (*imd, relish, pgrp-lc, pgrp-lb* and *pgrp-lbt* in adults) or eight days (*pgrp-lb* and *pgrp-lbt* in larvae) following dsRNA injection. Specificity of the *pgrp-lbt* inhibition was also checked by quantifying *pgrp-lbe* and *pgrp-lbi* transcript levels. A.U.: Arbitrary Units. The mean and standard error for five independent replicates are represented. Asterisks indicate a significant difference between two conditions based on a Welch's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **B:** PGRP-LB localization in larval tissues by immunostaining eight days after *pgrp-lb* RNAi treatment. A pre-immune serum was used as the negative control (lower panels). PGRP-LB-associated signal is observed strongly in the bacteriome of RNAi *gfp* individuals (right upper panel), and is lost in RNAi *pgrp-lb* individuals (left upper panel). This confirms the efficiency of the RNAi at the protein level, as well as the specificity of the serum. Blue: DAPI; green: PGRP-LB; red: autofluorescence. Bact: bacteriome. **C:** DNA construction for *pgrp-lbt*-specific dsRNA synthesis. The upper panel is a schematic representation for the construction. The lower panel is the sequence of the DNA construction. TM-domain represents the transcript portion encoding the transmembrane domain, only specific sequence of the *pgrp-lbt* transcript. TM-domain was duplicated based on two different fragments, while preserving specific extremities for PCR amplification (in green and red).

Table S1: Quantification of sugar and amino acid components in *S. pierantonius* PG after hydrolysis. Related to Figure 1. Components in red are typically found in DAP-type PG.

	Replicate 1 (nmol)	Replicate 2 (nmol)
Asp	0.71	0.28
Thr	0.26	0.09
Ser	0.78	0.27
Mur	**0.2**	**0.12**
Glu	1.18	0.54
Gly	1.21	0.39
Ala	0.85	0.44
Val	0.22	0.09
DAP	**0.26**	**0.14**
Ile	0.17	0.06
Leu	0.45	0.14
Tyr	0.11	0.01
Phe	0.18	0.05
GlcN	**0.4**	**0.17**
Lys	0.26	0.08
His	0.1	0.08
Arg	0.28	0.02

Table S2: Numbers of TCT-positive individuals, either the Malpighian tubules or the gut, after *pgrp-lb* or *pgrp-lbt* inhibition. Related to Figure 7. n corresponds to the total of individuals that were observed for each condition.

	RNAi *gfp* (n = 7)	RNAi *pgrp-lb* (n = 15)	RNAi *pgrp-lbt* (n = 6)
Malpighian tubules	0	7	2
Gut	0	3	0

Table S3: List of primers used for PCR, qPCR, dsRNA synthesis and plasmid constructions.

	Primers	Sequence (5'-3')
PCR	5-pgrp-lbi/t-pcr	TCCAGTAAGCAATCACGGTCG
	3-pgrp-lbi/t-pcr	CTGTTTTATACTCCTCGACGT
	5-pgrp-lbe-pcr	GTGTCTTGATTTGGGTAATATTC
	3-pgrp-lbe-pcr	TTACTGTTTTATACTCCTCGACGT
qPCR	5-relish	CGTGGGCTTGAACAAAAAGGA
	3-relish	GGTGTTGCCATGACCATCCA
	5-pgrp-lc	AGCATCACCACCCAACATTC
	3-pgrp-lc	TCACGGGTCCCATATATCCC
	5-imd	ATCCGGTAACCGAAGACGATA
	3-imd	ACAGTGGCTTTTTTAGTACCATTG
	5-coleoptericinA	GAATAGATACAACGGGGGTCA
	3-coleoptericinA	CTACCATCTGACACTTCCTC
	5-coleoptericinB	CTGATGTTGGCCACTATCGC
	3-coleoptericinB	CCATGCTGGTTCACTTCCAC
	5-sarcotoxin	AGTCACAAAGGAACAATTTTGG
	3-sarcotoxin	AGAAGCCGTCACGTCCGTTC
	5-mdh	CCAAGAACAACAAACAACCG
	3-mdh	CTACACCAGGGAAGATGTAT
	5-rpl29	TGGCCAAGTCCAAGAATCACA
	3-rpl29	TTCTTGGCGCTAGCTTGTCTT
	5-gapdh	AACTTTGCCGACAGCCTTGG
	3-gapdh	GCGCCCATGTATGTAGTTGG
	5-NuoCD	AATCGACGATCTCTTCACCG
	3-NuoCD	GAATTCGATCCCTTCGTCCT
	5-β-tubulin	CAACTCCATCATGAAGTGCG
	3-β-tubulin	CACATCTGTTGGAAGGTGGA
	5-pgrp-lbi	AGAGAAAAGAGTTGAATTAAATATAGTTGCGAACA
	3-pgrp-lbi	ATTGCATGTCATTTATACATTCCTC
	5-pgrp-lbt	TTACCCCAGGTTGCTCCAC
	3-pgrp-lbt	ATTGCATGTCATTTATACATTCCTC
	5-pgrp-lbe	GTGTCTTGATTTGGGTAATATTC
	3-pgrp-lbe	ATTGCATGTCATTTATACATTCCTC
RNAi	5-relish-T7	TAATACGACTCACTATAGGGCCAACATGGCGGATCCCATA
	3-relish-T7	TAATACGACTCACTATAGGGCGGGTGACGCTTAAACCATC
	5-pgrp-lc-T7	TAATACGACTCACTATAGGGAGCGCCATACATCGAAAACG
	3-pgrp-lc-T7	TAATACGACTCACTATAGGGTCACCGCCACTATACTTCTCAC
	5-imd-T7	TAATACGACTCACTATAGGGACACAGGAACAGTTTTCGTTCG
	3-imd-T7	TAATACGACTCACTATAGGGCGAATCCAATCCAGCAAGAACTG

	5-pgrp-lb-T7	TAATACGACTCACTATAGGGCCAGTCCCTTACGTCGTCAT
	3-pgrp-lb-T7	TAATACGACTCACTATAGGGTCTGTTTCTCGGACTTGCCT
	5-pgrp-lbt-T7	TAATACGACTCACTATAGGGCCCCAGGTTGCTCCACAT
	3-pgrp-lbt-T7	TAATACGACTCACTATAGGGAAATGTTGCACATATGATGACT
	5-gfp-T7	TAATACGACTCACTATAGGGCAAGGAGGACGGCAACATCC
	3-gfp-T7	TAATACGACTCACTATAGGGATTTTATGTTTCAGGTTCAG
DNA constructs	5_TM-domain_F1	CCCCAGGTTGCTCCACAT
	3_TM-domain_F1_ClaI	ATCGATGCGATGACTAACACTGCAC
	5_TM-domain_F2_ClaI	CCGATCGATTACCCCCAGAATTCCTGGAG
	3_TM-domain_F2_ClaI	CCCATCGATAAATGTTGCACATATGATGACT
	pgrp-lbi-S2-EcoRI-for	CCGGAATTCATGTCCAGTAAGCAATCACGGTCGA
	pgrp-lbi-S2-EcoRV-rev	CCGGATATCCCTGTTTTATACTCCTCGACGTAC
	pgrp-lbt-S2-EcoRV-for	CCGGATATCATGTCCAGTAAGCAATCACGGTCG
	pgrp-lbt-S2-XhoI-rev	CCGCTCGAGCTGTTTTATACTCCTCGACGTACT
	Fw_EGFP_KpnI	CGGGGACCTACTCCAGTATGGTGAGCAAGGGCGAGGA
	Rv_EGFP_EcoV	CCGGAATGAATTCTTGTACAGCTCGTCCATGC

Supplementary sequences: Sequences of the putative PGRP-LB proteins and transcripts identified in this study from three weevil species (*Sitophilus zeamais, Sitophilus oryzae* and *Sitophilus linearis*), from RNAseq data. Related to Figure 3. Protein sequences of the different isoforms were aligned for each species, in which the residues essential to enzymatic activity are shown in green, the transmembrane domain in red, the peptide signal in purple and the N-terminal region, specific to PGRP-LBi and -LBt without particular feature in grey. The specific PGRP-LBi and -LBt N-terminal sequences (grey and red) do not show homology with any sequence of the NCBI database.

***Sitophilus zeamais* PGRP-LB protein sequences**

```
Szeamais_PGRP-LBi      MSSKQSRSIVPTCECKFEKMEEIGEKKVELNIV---------------------------      33
Szeamais_PGRP-LBt      MSSKQSRSIVPTCECKFEKMEEIGEKKVELNIVPQVAPHYPQNSWRSLLTKSRIILIVTV      60
Szeamais_PGRP-LBe      ------------------------------------------------------MCYNSC      6

Szeamais_PGRP-LBi      -----------ANSFVCTDPEIVTRDEWGAKPPTGVENLTLPVPYVVIHHSYIPAACSTK      82
Szeamais_PGRP-LBt      LVIAVIICATFANSFVCTDPEIVTRDEWGAKPPTGVENLTLPVPYVVIHHSYIPAACSTK      120
Szeamais_PGRP-LBe      VLIWVIFTCHVANSFVCTDPEIVTRDEWGAKPPTGVENLTLPVPYVVIHHSYIPAACSTK      66
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Szeamais_PGRP-LBi      EECINDMQWMQNYHQQNNSWCDIGYNFAVGGDNRIYVGRGWTAVGAHAPRYNARSIGIVL      142
Szeamais_PGRP-LBt      EECINDMQWMQNYHQQNNSWCDIGYNFAVGGDNRIYVGRGWTAVGAHAPRYNARSIGIVL      180
Szeamais_PGRP-LBe      EECINDMQWMQNYHQQNNSWCDIGYNFAVGGDNRIYVGRGWTAVGAHAPRYNARSIGIVL      126
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Szeamais_PGRP-LBi      IGDWTEILPPESQMLAAKQLINMGIRDGYISENYKLIGHRQVRETECPGEALYKEIQTWP      202
Szeamais_PGRP-LBt      IGDWTEILPPESQMLAAKQLINMGIRDGYISENYKLIGHRQVRETECPGEALYKEIQTWP      240
Szeamais_PGRP-LBe      IGDWTEILPPESQMLAAKQLINMGIRDGYISENYKLIGHRQVRETECPGEALYKEIQTWP      186
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Szeamais_PGRP-LBt      HWIDNPSLDDEVIPVVPLDKEDLKPDPREDSGASLASEDNKIKRHSSTHKEESTSRSIKQ      300
Szeamais_PGRP-LBe      HWIDNPSLDDEVIPVVPLDKEDLKPDPREDSGASLASEDNKIKRHSSTHKEESTSRSIKQ      246
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PPESQMLAAKQLINMGIRDGYISENYKLIGHRQVRETECPGEALYKEIQT
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HKEESTSRSIKQ

>Szeamais_PGRP-LBt TRINITY_DN43557_c0_g1_i5

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LPVPYVVIHHSYIPAACSTKEECINDMQWMQNYHQQNNSWCDIGYNFAVG
GDNRIYVGRGWTAVGAHAPRYNARSIGIVLIGDWTEILPPESQMLAAKQL

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>Szeamais_PGRP-LBe TRINITY_DN43557_c0_g1_i3
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IRDGYISENYKLIGHRQVRETECPGEALYKEIQTWPHWIDNPSLDDEVIP
VVPLDKEDLKPDPREDSGASLASEDNKIKRHSSTHKEESTSRSIKQ

***Sitophilus oryzae* PGRP-LB protein sequences**

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Soryzae_PGRP-LBt     MSSKQSRSVPTRECQFEKMEEIEEQKVKLNIASQVASHYPQNSWRSLLTKSRIILIVTVL     60
Soryzae_PGRP-LBe1    -----------------------------------------------------MCYNICV     7
Soryzae_PGRP-LBe2    --------------------------------------------------------MICY     4
Soryzae_PGRP-LBe3    --------------------------------------------------------MISY     4

Soryzae_PGRP-LBi     ---------VANSFVCTDPEIVTRDEWGAKPPTDVENLTLPVPYVVIHHSYIPAACSTKE     82
Soryzae_PGRP-LBt     VIAVIICGTFANSFVCTDPEIVTRDEWGAKPPTDVENLTLPVPYVVIHHSYIPAACSTKE     120
Soryzae_PGRP-LBe1    LIWVILTCHVANSFVCTDPEIVTRDEWGAKPPTDVENLTLPVPYVVIHHSYIPAACSTKE     67
Soryzae_PGRP-LBe2    SLVSLYLFRVANSFVCTDPEIVTRDEWGAKPPTDVENLTLPVPYVVIHHSYIPAACSTKE     64
Soryzae_PGRP-LBe3    ILVILCYFTVANSFVCTDPEIVTRDEWGAKPPTDVENLTLPVPYVVIHHSYIPAACSTKE     64
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Soryzae_PGRP-LBe2    ECINDMQWMQKYHQQNNSWCDIGYNFAVGGDNRIYVGRGWTAVGAHAPRYNSRSIGIVLI     124
Soryzae_PGRP-LBe3    ECINDMQWMQKYHQQNNSWCDIGYNFAVGGDNRIYVGRGWTAVGAHAPRYNSRSIGIVLI     124
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Soryzae_PGRP-LBe1    GDWTDILPPESQMLAAKQLINMGIRDGYISENYKLIGHRQVRETACPGEALYKEIQMWPH     187
Soryzae_PGRP-LBe2    GDWTDILPPESQMLAAKQLINMGIRDGYISENYKLIGHRQVRETACPGEALYKEIQMWPH     184
Soryzae_PGRP-LBe3    GDWTDILPPESQMLAAKQLINMGIRDGYISENYKLIGHRQVRETACPGEALYKEIQMWPH     184
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Soryzae_PGRP-LBe1    WIPNPDEEVIPVVPLDKEDLKPDPREDPDASLASEDNEIKRHSSTDKDESTSRSIKHT       245
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PESQMLAAKQLINMGIRDGYISENYKLIGHRQVRETACPGEALYKEIQMW
PHWIPNPDEEVIPVVPLDKEDLKPDPREDPDASLASEDNEIKRHSSTDKD
ESTSRSIKHT
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IRDGYISENYKLIGHRQVRETACPGEALYKEIQMWPHWIPNPDEEVIPVV
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***Sitophilus linearis* PGRP-LB protein sequences**

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Slinearis_PGRP-LBe2      -----MINYIFAVLCYFNVVNSFVYPDPEIVTREEWCAKPPTSVEKLTLPVPYVVIHHSY      55
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Slinearis_PGRP-LBe1      IPAACYNKEECINAMQWMQKFHQQNRSWCDIGYNFAVGGDNRIYVGRGWTAVGAHAPLYN      120
Slinearis_PGRP-LBe2      IPAACYNKEECINAMQWMQKFHQQNRSWCDIGYNFAVGGDNRIYVGRGWTAVGAHAPLYN      115
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Slinearis_PGRP-LBe2      TKSMGIVLIGNWEDILPPESQMLVAKQLINMGIRGGYISKNYKLIGHRQVRETVCPGQAL      175
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Slinearis_PGRP-LBe1      YEEIQTWPHFTENPTLEIRRDVPVSLDKEHVKPDPREEFDARLSFENNQVRRRSSINKE       239
Slinearis_PGRP-LBe2      YEEIQTWPHFTENPTLEIRRDVPVSLDKEHVKPDPREEFDARLSFENNQVRRRSSINKE       234
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Supplementary references

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