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

Justin Maire, Carole Vincent-Monégat, Séverine Balmand, Agnès Vallier, Mireille Herve, Florent Masson, Nicolas Parishot, Aurélien Vigneron, Caroline Anselme, Jackie Perrin, Julien Orlans, Isabelle Rahiou, Pedro Da Silva, Marie-Odile Fauvarque, Dominique Mengin-Lecreulx, Anna Zaidman-Remy, and Abdelaziz Heddi

Abstract

Long-term intracellular symbiosis (or endosymbiosis) is widely distributed across invertebrates and is recognized as a major driving force in evolution. However, the maintenance of immune homeostasis in organisms chronically infected with mutualistic bacteria is a challenging task, and little is known about the molecular processes that limit endosymbiont immunogenicity and host inflammation. Here, we investigated peptidoglycan recognition protein (PGRP)-encoding genes in the cereal weevil Sitophilus zeamais’ association with Sodalis pierantonius endosymbiont. We discovered that weevil pgrp-lb generates three transcripts via alternative splicing and differential regulation. A secreted isoform is expressed in insect tissues under pathogenic conditions through activation of the PGRP-LC receptor of the immune deficiency pathway. In addition, cytosolic and transmembrane isoforms are permanently produced within endosymbiont-bearing organ, the bacteriome, in a PGRP-LC-independent manner. Bacteriome isoforms specifically cleave the tracheal cytotoxin (TCT), a peptidoglycan monomer released by endosymbionts. pgrp-lb silencing by RNAi results in TCT escape from the bacteriome to other insect tissues, where it chronically activates the host systemic immunity through PGRP-LC. While such immune deregulations did not impact endosymbiont load, they did negatively affect host physiology, as attested by a diminished sexual maturation of adult weevils. Whereas pgrp-lb was first described in pathogenic interactions, this work shows that, in an endosymbiosis context, specific bacteriome isoforms have evolved, allowing endosymbiont TCT scavenging and preventing chronic endosymbiont-induced immune responses, thus promoting host homeostasis.

Significance

Permanent infections with beneficial bacteria are widespread in nature and are believed to play a pivotal role in evolution. How hosts’ immune functions are regulated to maintain cooperative bacteria while preventing them from constantly activating the host immune system is a key question in understanding host–bacterial associations’ sustainability. In insects, beneficial bacteria are often confined within specialized host cells, the bacteriocytes, which avoid direct contact between the host’s immune system and bacteria. Here, we report an additional mechanism that prevents bacteria-released immunogenic molecules (i.e., peptidoglycan fragments) from escaping the bacteriocytes. We show that two peptidoglycan-cleaving enzymes are specifically produced in the bacteriocytes, where they cleave bacterial peptidoglycan into nonimmunogenic fragments, thereby preventing continuous and damaging host systemic immune activation.


The authors declare no conflict of interest.

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Data deposition: Datasets have been deposited in the NCBI Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra (transcriptome datasets (accession nos. SRP149413 and SRP149424); cDNA sequences (accession nos. MH423625 and MH423627–MH423629).
Candidatus Sodalis pierantonius, hereafter referred to as S. pierantonius (8–10) (Fig. 1 A and B). S. pierantonius was acquired recently (28,000 y ago), following symbiont replacement within the Dryophthoridae family, and retains similar genomic features with free-living bacteria (11–13). It encodes genes necessary for MAMP synthesis, including PG (9), and its experimental injection in the insect hemolymph elicits a systemic immune response (14), making this model system particularly relevant to study host immune adaptations to endosymbiont housing. We previously showed that the bacteriome selectively produces one antimicrobial peptide (AMP), coleopterin A (Col-A), which targets endosymbionts and prevents their escape from the bacteriocyes (15). Nonetheless, bacterial PG fragments could exit the bacteriocytes and trigger a systemic immune response. While it has been demonstrated that PG is able to cross membranes and epithelial barriers in other organisms (16–21), mechanisms that avoid PG release from insect bacteriomes remain unknown. PG is detected through host PG recognition proteins (PGRPs), a family of conserved proteins believed to have evolved from bacteriophage T7 lysozymes able to cleave PG (22). In animals, PGRPs have evolved toward three main functions in host–bacteria interactions: immunity activator upon PG recognition; immune pathway modulator, notably through PG cleavage into nonimmunogenic fragments; and immune effector through bactericidal activity (23, 24).

In Drosophila, PGRPs are essential in the activation and regulation of the immune deficiency (IMD) pathway, in response to Gram-negative bacteria (24). In particular, the transmembrane receptor PGRP-LC recognizes circulating polymeric PG as well as a monomeric fragment, the tracheal cytotoxin (TCT), from Gram-negative bacteria (25, 26), while TCT located within insect cells is detected by the intracellular PGRP-LR (21, 27, 28) (SI Appendix, Fig. S1A). PG detection activates the IMD pathway, resulting in the nuclear translocation of NF-kB transcription factor Relish and AMP-encoding gene up-regulation (29). PGRP-LB negatively modulates the IMD pathway by cleaving bacterial PG into nonimmunogenic fragments (20, 30) (SI Appendix, Fig. S1A). We recently demonstrated that imd and relish are required for immune activation upon TCT injection in cereal weevils, and for endosymbiont-specific immune response within the bacteriome (31). However, the mechanism by which S. pierantonius activates the host local immunity in the bacteriome, without chronically triggering a systemic immune response, remains unknown.

Here, we have studied the function of pgrp genes with regards to symbiosis and host immunity in the cereal weevil S. zeamais. We show that pgrp-le is absent, and that pgrp-lc is essential for circulating-PG recognition and consequent IMD pathway activation. Remarkably, pgrp-lc is not involved in endosymbiont recognition within the bacteriome. Molecular investigations showed, however, that the bacteriome expressed forms of the pgrp-lb gene specifically cleave within the bacteriome symbiont-released TCT, preventing its dissemination to insect tissues and subsequent chronic immune activation via PGRP-LC, which is detrimental to the weevil host but not to its associated endosymbionts.

Results and Discussion

S. pierantonius Produces a Diaminopimelic Acid-Type PG That Triggers the Weevil Systemic Immune Response. We first sought to understand how S. pierantonius activates the host systemic immune response when injected into the insect hemolymph. Because S. pierantonius has conserved the genes required for PG synthesis (9), we hypothesized that such a molecule might mediate endosymbiont recognition through host immune receptors. PG is composed of glycan chains, formed by an alternation of N-acetylmuramic acid (GlcNAc) and N-acetylmuramic acid (MurNAc), and cross-linked by short peptides. Peptide composition changes between bacteria, depending on the amino acid being incorporated in the third position by the MurE enzyme [a lysine in Gram-positive cocci, and a diaminopimelic acid (DAP) in Gram-positive bacilli and Gram-negative bacteria (32, 33)]. S. pierantonius MurE possesses a four-amino acid consensus sequence found in DAP-incorporating MurE (Fig. 1C) (34). Biochemical analyses confirmed that S. pierantonius does synthesize DAP-type PG (Fig. 1D and SI Appendix, Table S1).

DAP-type PG is an elicitor of the IMD pathway in insects, so we wondered if such an activation occurs in S. zeamais. We injected larvae with polymeric DAP-type PG, TCT, or MurNAc-tripeptide-DAP purified from Escherichia coli (SI Appendix, Fig. S1B), and monitored the transcript steady-state levels of three AMP-encoding genes commonly used as an IMD pathway readout in Sitophilus (31, 35, 36), namely coa, coleopterin B (colB), and sarcotoxin. We chose to study the larval instar in which, in contrast to adults, the endosymbiont load remains relatively stable and the bacteriocytes do not exhibit any cellular changes (37). Both polymeric DAP-type PG and TCT strongly induced systemic AMP-encoding gene expression compared with PBS or MurNAc-tripeptide-DAP injection (Fig. 2C and SI Appendix, Fig. S1C). This induction peaks at 6-h postinjection, a time point we used for further experiments. Induction of a systemic immune response by polymeric DAP-type PG and monomeric TCT is consistent with previous studies in cereal weevils (31) and Drosophila (38). We therefore hypothesized that S. pierantonius TCT could trigger the S. zeamais immune system.
Indeed, in Gram-negative bacteria, PG is—most of the time—hidden from host immune receptors under the lipopolysaccharide-containing outer membrane layer, while the monomeric TCT is accessible due to its release during bacterial proliferation and cell wall recycling (39). Moreover, TCT is involved in several pathogenic infections, including with *Bordetella pertussis* and *Neisseria gonorrhoeae*, where it triggers epithelial shedding (40, 41). Finally, TCT was shown to be essential for symbiont colonization in the mutualistic association between the squid *Euprymna scolopes* and *Vibrio fischeri* (42).

**PGRP-LC Regulates Systemic Immunity, but Not the Bacteriome Immune Program.** To understand how TCT is recognized by the weevil’s immune system, we next studied the PGRP receptors in *S. zeamais*, PGRP-LC and -LE ([SI Appendix](#), Fig. S1A). Remarkably, no *pgrp-le* transcript has been identified in *S. zeamais* transcriptomic data. Interestingly, *pgrp-le* is also absent from the genome of the tsetse fly (43), another symbiotic insect that harbors the endosymbiont *Wigglesworthia glossinidia*. The lack of intracellular TCT receptors in insects harboring endosymbionts may be a common feature that avoids the induction of local immune responses against endosymbionts. However, we did identify in *S. zeamais* a *pgrp-le* ortholog and assessed its function with RNA interference (RNAi). To avoid interference with the bacteriome-specific immunity (7, 35), we separated larvae into two different samples: the bacteriome and the rest of the body, hereafter referred to as the “carcasses.” Induction of systemic AMP-encoding gene expression by TCT was significantly lower in RNAi *pgrp-le* carcasses, compared with controls (Fig. 2B). Thus, PGRP-LC is necessary for extracellular TCT detection and the ensuing systemic immune response in *S. zeamais*. Similar results were obtained in dissected bacteriomes ([SI Appendix](#), Fig. S1D), suggesting that the bacteriome immunocompetency to exogenous challenges reported in a previous study (35) is also PGRP-LC-dependent. Intriguingly, AMP-encoding gene expression in the bacteriome, without TCT injection, did not significantly change following *pgrp-le* inhibition (Fig. 2C), suggesting that the bacteriome immune response to intracellular stimuli (i.e., endosymbionts) is not initiated by PGRP-LC, despite being IMD/Relish-dependent (Fig. 2C) (31). Hence, *S. zeamais* does not seem to recognize intracellular TCT within the bacteriome, all the while being able to detect circulating TCT outside of the bacteriome, both systemically and locally, enabling the insect to mount an efficient immune response against bacterial intruders. Nonetheless, even in the absence of intracellular TCT detection inside the bacteriome, based on the strong metabolic exchanges between *S. pierantonius* and *S. zeamais* (9, 37, 44–46), we hypothesized that endosymbiont TCT might hitchhike with metabolites during their export from the bacteriome. Thus, symbiont-released TCT may exit the bacteriome and induce a systemic PGRP-LC-dependent immune response.

**Weevil *pgrp-lb* Expresses Two Bacteriocyte Isoforms and a Secreted One Through Alternative Splicing.** In *Drosophila*, down-regulation of PGRP-LC–mediated IMD pathway activation is notably accomplished by PGRP-LB ([SI Appendix](#), Fig. S1A), which cleaves DAP-type PG into nonimmunogenic fragments through an amidas activity, hence, modulating the response to both pathogens and gut commensals (20, 30, 47). Interestingly, a *pgrp-lb* ortholog in *S. zeamais*, named *wpggp1* in previous studies, is highly expressed in the bacteriome (45, 48). Immunostaining experiments during weevil development showed that PGRP-LB could be detected both intra- and extracellularly (Fig. 3 A and B). During the embryonic stages, PGRP-LB seems to be extracellular in the fat body (Fig. 3A). At the larval and adult stages, PGRP-LB signal seems to be found extracellular in the fat body and, remarkably, intracellular within gut epithelial cells and the bacteriocytes ([Fig. 3B and SI Appendix](#), Fig. S2). The strong production of PGRP-LB in the bacteriocytes presumes a potential function linked to endosymbiosis. We sequenced the transcriptome of *S. zeamais* whole adults and subsequently discovered three *pgrp-lb* splice variants, a finding that was further confirmed by RT-PCR ([Fig. 3 C and D and SI Appendix, Supplementary Sequences](#)). These transcripts encode three different proteins that share a predicted amidase domain. Interestingly, these isoforms are predicted to have distinct subcellular locations, according to which we named them PGRP-LBi for intracellular, *pgrp-lb* isoform, and PGRP-LBc for secreted (Fig. 3D)).

![Fig. 2.](image.png)

**Fig. 2.** Circulating TCT, but not intracellular *S. pierantonius*, triggers a *pgrp-lc*-dependent immune response. (A) Systemic AMP-encoding gene expression kinetics following PG injection. *colA*, *colB*, and *sarcotoxin* expression were measured in whole larvae by qRT-PCR 2, 6, 12, or 24 h after injections of either PBS (gray), *E. coli* polymeric DAP-type PG (green), or *E. coli*–derived TCT (blue). TCT and polymeric DAP-type PG both significantly induce AMP expression in weevil larvae, based on the analysis of a generalized linear model. (B and C) AMP-encoding gene expression following *pgrp-lc* inhibition by RNAi. *colA*, *colB*, and *sarcotoxin* expression were measured in carcasses by qRT-PCR 6 h after either PBS or TCT injection (B), or in the bacteriome under standard conditions (C) 6 d following *pgrp-lc* or *relish* dsRNA injection. AMP expression does not increase following TCT injection when *pgrp-lc* is inhibited. However, AMP-encoding gene basal, symbiont-dependent expression in the bacteriome is not affected by *pgrp-lc* inhibition. By comparison, *relish* inhibition abolishes AMP-encoding gene expression in the bacteriome, as was previously shown (31). Asterisks indicate a significant difference between two conditions based on a Welch’s *t* test. The mean and SE for five independent replicates are represented. A.U., arbitrary units. n.s., nonsignificant; *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 3. S. zeamais pgrp-lb encodes two symbiosis related protein isofoms and one secreted isofom. (A and B) Immunostaining of PGRP-LB in embryos (A) and larval tissues (B). A preimmune serum was used as negative control (Lower). PGRP-LB-associated signal seems extracellular in the embryo fat body (A), and the larval fat body (B, Right). Intracellular signal is observed strongly in the bacteriome (B, Left), and in the gut (B, Center). Blue: DAPI; green: PGRP-LB; red: autofluorescence. Bact, bacteriome; Fb, fat body. (C) Schematic representation of the three alternatively spliced pgrp-lb transcripts and their occurrence in three weevil species: S. zeamais and S. oryzae that house the endosymbiont S. prierontius, and S. linearis that is naturally asymbiotic. Three transcripts are detected in the symbiotic species, while only pgrp-lbe (encoding the secreted isoform) is found in S. linearis. The green color indicates the common sequence between all three transcripts and includes the predicted enzymatic domain. The purple color indicates the sequence encoding the predicted signal peptide, specific to pgrp-lbe. The red color indicates the sequence encoding the predicted transmembrane domain, specific to pgrp-lbt. The gray color indicates the common sequence between pgrp-lbi and -lbt. The specific PGRP-LBI and -LBT N-terminal sequences (gray and red) do not show similarity with any sequence of the National Center for Biotechnology Information (NCBI) database. (D) Agarose gel electrophoresis showing the three pgrp-lb transcripts amplified by RT-PCR from S. zeamais’ total cDNA. Arrowheads point at the three bands corresponding to the three pgrp-lb transcripts. (E) pgrp-lb transcript quantification in larval tissues. pgrp-lbi, pgrp-lbt, and pgrp-lbe transcripts were quantified by qRT-PCR with specific sets of primers. The mean and SE of five independent replicates are represented. pgrp-lbi levels are 5, 340, and 27 times higher in the bacteriome than in the gut, the fat body, and the whole body, respectively; pgrp-lbt levels are 8, 1,100, and 100 times higher in the bacteriome than in the gut, the fat body, and the whole body, respectively.

PGRP-LBt for transmembrane, and PGRP-LBe for extracellular (Fig. 3C and SI Appendix, Supplementary Sequences).

Consistent with the immunostaining data, quantitative RT-PCR experiments revealed high amounts of pgrp-lbi and -lbt transcripts in the bacteriocytes, contrasting with the low amounts of pgrp-lbe in all tissues (Fig. 3E). To address the intracellular and transmembrane nature of the bacteriocyte PGRP-LB variants, we transfected Drosophila S2 cells with V5-tagged PGRP-LBi and PGRP-LBt. Immunostaining showed intracellular versus transmembrane signal for PGRP-LBi and -LBT, respectively (SI Appendix, Fig. S3). Using a double GFP-V5–tagged construction and immunostaining with or without membrane permeabilization, we further showed that PGRP-LBt is inserted in both orientations within Drosophila cell membranes (SI Appendix, Fig. S3). PGRP-LBt could either be initially inserted in both orientations, or it could be able to change its topology within membranes, as was notably described in E. coli following membrane lipid composition changes (49). This further strengthens the transmembrane localization of PGRP-LBt, and suggests that its predicted amidase activity may occur on both sides of the membranes, although the situation might be different in the bacteriocyte, where interactions with weevil proteins expressed in this specialized cell could stabilize PGRP-LBt orientation.

Intriguingly, N-terminal sequences specific to PGRP-LBi and -LBT do not show similarity with any known sequence (Fig. 3C and SI Appendix, Supplementary Sequences). To further understand the origin of these transcripts within the Sitophilus genus, and to determine the impact of endosymbiosis in pgrp-lb evolution, we sequenced the transcriptome of an additional symbiotic species, the cereal weevil Sitophilus oryzae that also houses S. prierontius, and of a naturally non symbiotic species, Sitophilus linearis (13). While all three pgrp-lb transcripts were expressed in S. oryzae, only the pgrp-lbe transcript was found in the non symbiotic S. linearis transcriptome (Fig. 3C and SI Appendix, Supplementary Sequences). Thus, pgrp-lbi and -lbt splice variants might be the result of an endosymbiosis–driven evolution of the pgrp-lb gene structure or regulation. These findings point toward a specific function for PGRP-LBi and -LBT in host–symbiont interactions. It is noteworthy that three PGRP-LB isoforms with distinct localization (extracellular or intracellular) have also recently been reported in the Drosophila model, in which they show distinct local and systemic immune modulation to the presence of gut commensal bacteria (47). Therefore, pgrp-lb subfunctionalization through alternatively spliced variants and differential subcellular localization could be viewed as an evolutionary molecular shaping of this gene family to the distinct constraints present in fruit flies and weevils (i.e., gut extracellular commensals versus endosymbionts residing inside specialized cells).

Weevil pgrp-lb Transcripts Are Differentially Regulated. Because pgrp-lbi and -lbt are specifically expressed in bacteriocytes, we next explored how these variants are locally regulated within the bacteriome organ, compared with insect systemic immunity. We show that pgrp-lbi and -lbt transcript levels are not affected in the bacteriome following PBS or TCT injection (Fig. 4D). In the carcass, however, both pgrp-lbi and -lbt transcript levels increased similarly following either PBS or TCT injection (Fig. 4D), indicating that pgrp-lbi and -lbt transcript levels are affected by
transcript levels increased significantly in the bacteriome and carcasses by qRT-PCR. Quantification was performed on naive individuals or 6 h after PBS or TCT injection. pgrp-lbe transcript levels are increased in all tissues following TCT injection, while pgrp-lbi and -lbt transcript levels remain stable. Arbitrary units are expressed as log-linear values. (B-D) Assessment of the IMD pathway implication in the regulation of pgrp-lb expression. Six days following imd (B), relish (C), or pgrp-lc (D) dsRNA injection, pgrp-lbi and pgrp-lbt transcript levels were quantified in the bacteriome (Left), and pgrp-lbe transcript level was quantified in the bacteriome and carcass, 6 h after PBS or TCT (Center and Right). pgrplb transcript levels are IMD- and Relish-dependent, but not PGRP-LC-dependent. The mean and SE 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).

**Weevil PGRP-LB Enzymatic Domain Has Evolved Specificity Toward TCT.** Because PGRP-LBi and -Lbt are strongly produced in the bacteriocytes, we sought to understand their precise role by characterizing their enzymatic activity. The prediction of an N-acetylmuramoyl-l-alanine amidase activity that cleaves PG relies on the presence of five amino acid residues, conserved across catalytic PGRPs and required for the zinc-binding–dependent amidase activity (Fig. S4) (S1). To determine whether S. pierantonius DAP-type PG is cleaved by the weevil PGRP-LB enzymatic domain, we analyzed the amidase activity of all three isoforms, PGRP-LBi, against polymeric DAP-type PG, MurNAc-tripeptide-Lys, MurNAc-tripeptide-DAP, and TCT purified from culturable bacteria (SI Appendix, Fig. S1B). We show that the weevil PGRP-LB enzymatic domain displays a strong specificity toward TCT (Fig. S5 B). Activities against DAP-type PG, MurNAc-tripeptide-Lys, and MurNAc-tripeptide-DAP were at least 18 times lower (Fig. S5 B). These data contrast with the Drosophila PGRP-LB activity, which is versatile for polymeric and monomeric DAP-type PG, and suggest that weevil PGRP-LB has diverged, probably under the evolutionary pressure of endosymbiosis. Recognition of PG by the amidase activity relies on three conserved amino acid residues, which differ between Lys- and DAP-type binding PGRPs (24). Interestingly, one of these amino acids is not conserved in the weevil PGRP-LB, which could account for TCT specificity (Fig. S5 C). TCT is a mediator of host–bacteria interactions in pathogenesis and mutualism. Modification of the bacterial TCT recycling machinery is known to modulate TCT release into the host.*
For example, in *Neisseria* species, the inner membrane permease AmpG ensures PG fragment recycling and limits the amount of TCT secreted (52, 53). Impaired AmpG activity correlates with a stronger virulence (53). Remarkably, we found that *ampG* is among the genes that were shown to be pseudogenized in *S. pierantonius* (9), suggesting that it may shed high quantities of TCT into the host. The PGRP-LB–specific amidase activity toward TCT could have emerged as an adaptive feature along host–symbiont coevolution, allowing the host to cope efficiently with symbiont-released TCT.

**Weevil *pgg*-*lb* Prevents Systemic Immune Activation by Endosymbionts.**

Our data strongly point to an endosymbiosis-driven evolution of *pgg*-*lb*, notably in terms of gene regulation and protein enzymatic activity; hence, we next evaluated *pgg*-*lb* function in host–symbiont interactions. To this end, we inhibited simultaneously all three *pgg*-*lb* transcripts by RNAi in larvae and quantified AMP-encoding gene transcription to associations with bacteria.

Weevil *pgg*-*lb* expression also significantly increased in carcasses of RNAi *pgg*-*lb* or RNAi *pgg*-*lt* adults (Fig. 6F), but in a much stronger extent than in larvae, consistent with the higher symbiont load. Beyond immunity, we hypothesized that such strong deregulations during a crucial developmental period might affect insect biological processes. Consequently, we analyzed the impact of *pgg*-*lb* inhibition on immune abilities (57). The impact observed on egg production reduces immune abilities (57). The impact observed on egg production could also be a direct deleterious effect of IMD pathway overactivation, which promotes apoptosis in *Drosophila*’s wing discs (58) and gut (59).

Weevil immune overactivation following *pgg*-*lb* inhibition impaired host physiological functions but did not impact endosymbiont load (SI Appendix, Fig. S44). We therefore conclude that in *S. zeamais*, PGRP-LB bacterioyte-isofoms do not act per se in endosymbiont preservation from the host immunity (i.e., what some authors refer to as “tolerance”), but in preventing chronic host inflammation due to endosymbiont presence. This contrasts with the tsetse fly in which *pgg*-*lb* inhibition leads to endosymbiont load decrease (50, 55), attesting to the crucial role of PGRP-LB in endosymbiont immune tolerance in this model. *pgg*-*lb* inhibition also results in an impaired fertility in female tsetse (55), which in this model could be the consequence of either resource competition with the overly activated immune system, or more probably of the decreased symbiotic population that is essential for host fertility (60).

**PGRP-LB Isoforms Prevent Endosymbiont TCT from Reaching the Host Systemic Immune System.** To pinpoint the molecular basis of AMP up-regulation in RNAi *pgg*-*lb* individuals, we simultaneously inhibited *pgg*-*lb* and *pgg*-*lc* expression, because PGRP-LC regulates AMP expression in the carcass (Fig. 2B). We show that....

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### Table A

<table>
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<th>Specific activity (nmol.min⁻¹.mg⁻¹)</th>
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<td>TCT</td>
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### Table C

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</table>
RNAi against *pgpr-lc* rescued the *pgpr-lb* RNAi phenotype (Fig. 7A), indicating that the systemic immune activation upon *pgpr-lb* inhibition is mediated by the receptor GPRP-LC. The presence of PG in the carcass could result from either endosymbionts or PG fragments escaping the bacteriome. However, FISH experiments with a specific probe directed against *S. pierantonius* did not reveal any symbiont outside of the bacteriome in RNAi *pgpr-lb* insects (*SI Appendix*, Fig. S4B). To strengthen these historical results, we show that PGRP-LBi did not display any bactericidal activity against the Gram-negative bacterium *E. coli* in vitro (*SI Appendix*, Fig. S4C), and that, as mentioned above, global endosymbiotic density was not affected by *pgpr-lb* RNAi (*SI Appendix*, Fig. S4A), indicating that PGRP-LB does not directly control symbiont populations. Furthermore, *colA* expression, the disruption of which results in endosymbiotic escape (15, 31), was not affected in the bacteriome by *pgpr-lb* inhibition (*SI Appendix*, Fig. S4D and E), suggesting that PGRP-LB does not indirectly control endosymbionts either. Overall, we demonstrate that systemic immune activation following *pgpr-lb* inhibition is not due to symbiont escape from the bacteriome.

To attest that the immune activation following *pgpr-lb* inhibition is due to TCT escape from the bacteriome organ, we developed a tool for in situ TCT detection. Based on the specific TCT binding ability of the *Drosophila* PGRP-LE recognition domain (27, 61), we constructed a V5-tagged recombinant PGRP-LE to use as a TCT detector by immunostaining (*SI Appendix*, Fig. S5). Remarkably, individuals treated with RNAi *pgpr-lb* or RNAi *pgpr-lbt* exhibited strong punctuated signal in the gut epithelia and Malpighian tubules (Fig. 7B and *SI Appendix*, Fig. S6 and Table S2). Malpighian tubules endorse detoxifying and excretory functions in insects, and are known to internalize TCT via oligopeptide carriers in *Drosophila* (16, 27).

These findings show that PGRP-LB specifically cleaves symbiotic TCT, preventing its dissemination out of the bacteriome and the subsequent activation of a PGRP-LC–dependent systemic immune response. Several mechanisms allowing PG to enter eukaryotic cells have been described in pathogenic interactions, including bacterial pore-forming toxins (62), outermembrane vesicles (63), and oligopeptide carriers (16). We hypothesize that similar mechanisms may permit PG released by intracellular symbionts to exit bacteriocytes. The strong vesicular network previously described in bacteriocytes and suggested to mediate host–symbiont metabolic exchanges (45, 64) could also participate in TCT transfer outside of the bacteriocytes. Based on the subcellular locations of PGRP-LBi and -Lbt, we propose that these bacteriome-specific isoforms cleave TCT both in the cytosol and in export vesicles, preventing direct contact between TCT and the systemic immunity receptor PGRP-LC. We also speculate that PGRP-LBe is able to cleave any TCT that might still escape the bacteriome, as is suggested by *pgpr-lbe* transcript level increase following specific *pgpr-lbt* transcript inhibition (*SI Appendix*, Fig. S7A).

Since TCT identification as the main toxin released by *B. pertussis* and *N. gonorrhoeae*, and as the causative agent of the whooping cough and gonorrhea (40, 41), TCT has been shown to be a major microbe–host signaling molecule both in pathogenic
and mutualistic interactions. Not only does TCT trigger host immunity, but it also widely affects host behavior (65) and morphogenetic processes (42, 66). Notably, in the beneficial association between the squid E. scolopes and V. fischeri, a TCT-cleaving PGRP is produced only after the symbiont-released TCT has triggered the symbiotic light organ morphogenesis (67). TCT could therefore impact other functions than immunity in the association between cereal weevils and S. pierantonius, functions that remain to be explored.

In conclusion, we have characterized host-specific local immune barriers that complement morphogenetic adaptations (bacteriocytes) and ensure immune homeostasis by preventing a chronic and detrimental host immune activation. While S. pierantonius CoA-dependent seclusion in bacteriocytes prevents endosymbionts from directly triggering host immunity, the PGRP family plays an equally important role by limiting symbiotic TCT-mediated local and systemic immune activation. We show that TCT is most likely not detected within the bacteriome, due to the absence of appropriate receptors, while its dissemination throughout host tissues and subsequent recognition by systemic receptors is limited through PGRP-LB local action in the bacteriome organ. Weevil pgrp-lb has evolved several features specific to endosymbiont-derived MAMP tolerance, including an increased enzymatic specificity against TCT, and the specific subcellular distribution of two isoforms within the bacteriocytes via the acquisition of novel sequences. This indicates how the PG-cleaving function has evolved from a presumed of-fensive role in phage T7 lysozymes to sustaining host homeostasis and symbiotic structures, but also shapes existing host immune genes and implements novel mechanisms that favor symbiont persistence without compromising the host’s defenses against pathogens.

Materials and Methods

See SI Appendix for additional materials and methods.

Biological Material and Sample Preparation. S. zeamais and S. oryzae weevils are reared on wheat grains at 27.5 °C and at 70% relative humidity. The Lagoa (S. zeamais) strains were chosen in this work because they are free of any facultative symbionts, including Wolbachia, and harbor only S. pierantonius. S. linearis individuals were collected in Niamey (Niger) and reared on tamarind seeds at 27.5 °C and at 70% relative humidity. Aposymbiotic insects were obtained as previously described (54). The apsymbiotic status was confirmed by PCR and histology experiments. Insect organs (bacteriomes, guts, ovaries) were dissected in diethylpyrocarbonate-treated Buffer A (25 mM KCl, 10 mM MgCl₂, 250 mM Sucrose, 35 mM Tris-HCl, pH = 7.5). For RNA extraction, at least five organs or whole individuals per condition were pooled and stored at −80 °C and each sampling was independently repeated five times. For DNA extraction, single whole individuals were stored at −80 °C and each sampling was independently repeated eight times.

PG Injections. PG fragments were produced and purified from E. coli as previously described (38). For TCT and MurNAc-tripeptide-DAP, 55 nL at a 0.2 mM concentration were injected into the hemolymph using a Nanoject II (Drummond). For whole DAP-type PG, after 5 min of sonication, 55 nL at 1 mM were injected. Sterile PBS was used as a negative control. Individuals were recovered 2, 6, 12, or 24 h after injection.

dsRNA Synthesis and Injection. dsRNA was prepared as described previously (68). Primers used for T7 DNA fragments are listed in SI Appendix, Table S3 and were designed to amplify a fragment from 200 bp to 300 bp. Next, 50 ng of dsRNA were injected into the hemolymph of third-instar larvae or 1-d-old adults with a Nanoject II (Drummond). dsRNA targeting the gfp gene was injected as a negative control. When two genes were targeted at once, 50 ng of each dsRNA were injected, and 100 ng of gfp dsRNA were injected as a negative control. Individuals were then kept on wheat flour, for 6 or 8 d, at a controlled temperature (27.5 °C) and relative humidity (70%). Efficiency of the RNAi was then checked by qRT-PCR (SI Appendix, Fig. S7A) and by immunostaining in the case of pgrp-lb inhibition (SI Appendix, Fig. S7B).

Total RNA Extraction and Reverse-Transcription. Total RNA from whole larvae or carcasses was extracted with TRizol reagent (ThermoFisher Scientific).

![Image](https://via.placeholder.com/150)
following the manufacturer’s instructions. RNA was incubated with 1 U/µg of RQ1 RNase-free DNase (Promega) for 30 min at 37 °C. Total RNA from bacteriomes, guts and fat body was extracted using RNAqueous Micro (Ambion), which allows for a better RNA yield from small tissue samples. After purification, the RNA concentration was measured with a Nanodrop spectrophotometer (ThermoFisher Scientific) and RNA quality was checked using agarose gel electrophoresis. Reverse-transcription into the first strand cDNA was carried out using the iScript cDNA Synthesis Kit (Bio-Rad).

**Real-Time qRT-PCR Transcript Quantification.** The quantification was performed with a LightCycler instrument using the LightCycler Fast Start Master DNA, RNA and Dna polymerase containing MurNAc-tripeptide as substrates (0.1 mM) produced with a LightCycler instrument using the LightCycler Fast Start DNA Real-Time qRT-PCR Transcript Quantification.

After purification, the RNA concentration was measured with a Nanodrop buffer with 50 mM Tris·HCl pH 7.5, 150 mM NaCl, 5 mM GSH, and 0.5 mM EDTA. The final samples were then buffer-exchanged by a standard dialysis method versus PBS pH 7.5 at a final concentration of 0.13 mg/mL (purity 90%) (ProteoGenix).

**Biochemical Assays.** PGRP-LB enzymatic activity was measured in 50-µL reaction mixtures containing 50 mM ethanolamine buffer, pH 9.6, 2.5 mM zinc sulfate, and unlabeled or radiolabeled forms of DAP-type PG, TCT, or Lys- and DAP-containing MurNAc-tripeptide as substrates (0.1–0.5 mM range). Radiolabeled PG and peptide fragments were obtained as previously described (20, 38). After incubation for 30 min (PG fragments) or several hours (PG) at 37 °C, the substrates and reaction products were separated by HPLC on a Nucleosil 100 C18 column (250 x 4.6 mm; Alltech France) using a gradient of methanol from 0 to 20% in 50 mM sodium phosphate buffer, pH 4.3, for elution, at a flow rate of 0.6 mL/min. Peaks were detected at 207 nm and the radioactivity was detected and quantitated with a flow detector as described in Zaidman-Rémy et al. (20). Digestion of the whole PG polymer by PGRP-LB released tetra- and octamers as main products and digestion of its MurNAc-tripeptide and TCT fragments released tri- and tetrapeptide, respectively. The amounts of peptides thus released were quantitated and used to determine the specific enzymatic activity of the PGRP-LB.

**Construction, Expression and Purification of a Drosophila PGRP-LE Recognition Domain Fusion with VS Epitope**. The gene segment coding for the PGRP coding domain (residues 173–345) of Drosophila PGRP-LE (Accession no. C9VXM9) was chemically synthesized with optimization for E. coli expression (ProteoGenix). The sequences coding for a 6His-tag and a V5-tag were added in 5’/3’er position in fusion with the ORF. This cDNA sequence was cloned in an E. coli p77 expression vector. The sequence of the expected protein produced is illustrated in SI Appendix, Fig. S5 A and B.

**TCT Localization Using a Drosophila PGRP-LE Recognition Domain Fusion with VS Epitope**. The same protocol as the one used for PGRP-LB immunostainings was used, using the fusion PGRP-LE-VS diluted either 1:100 or 1:200 incubated overnight at 4 °C, a mouse anti-VS antibody diluted 1:500 (ThermoFisher Scientific) incubated for 1 h at room temperature, and an Alexa Fluor 647 goat anti-mouse IgG diluted 1:1,000 (ThermoFisher Scientific) incubated for 1 h at room temperature. Aposymbiotic larvae were used as a negative control to find the dilution that yielded the least possible background signal in individuals that do not contain any bacteria that could release TCT (SI Appendix, Fig. S5D). The injected larvae were used as a positive control: 55 nl of a 0.2 mM purified TCT solution were injected, and larvae were recovered, fixed 1 h later and processed as described in SI Appendix, Supplementary Materials and Methods. The signal observed in the fat body and its decrease proportional to PGRP-LE-VS concentration confirmed the capacity of our construction to detect TCT in situ (SI Appendix, Fig. S5C). Images were acquired the same way as described in SI Appendix, Supplementary Materials and Methods.

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8. Heddi A, Khalturin K, Bonnot G, Nardon P (1998) Molecular characterization of the principal symbiotic bacteria of the weevil Sitophilus oryzae: Aposymbiotic larvae were used as a negative control to find the dilution that yielded the least possible background signal in individuals that do not contain any bacteria that could release TCT (SI Appendix, Fig. S5D). The injected larvae were used as a positive control: 55 nl of a 0.2 mM purified TCT solution were injected, and larvae were recovered, fixed 1 h later and processed as described in SI Appendix, Supplementary Materials and Methods. The signal observed in the fat body and its decrease proportional to PGRP-LE-VS concentration confirmed the capacity of our construction to detect TCT in situ (SI Appendix, Fig. S5C). Images were acquired the same way as described in SI Appendix, Supplementary Materials and Methods.