Host *PGRP* Gene Expression and Bacterial Release in Endosymbiosis of the Weevil *Sitophilus zeamais*

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Intracellular symbiosis (endosymbiosis) with gram-negative bacteria is common in insects, yet little is known about how the host immune system perceives the endosymbionts and controls their growth and invasion without complete bacterial clearance. In this study, we have explored the expression of a peptidoglycan recognition protein gene of the weevil *Sitophilus zeamais* (*wPGRP*); an ortholog in *Drosophila* (i.e., *PGRP-LB*) was recently shown to downregulate the Imd pathway (A. Zaidman-Remy, M. Herve, M. Poidevin, S. Pili-Floury, M. S. Kim, D. Blanot, B. H. Oh, R. Ueda, D. Mengin-Lecreulx, and B. Lemaitre, *Immunity* 24:463–473, 2006). Insect challenges with bacteria have demonstrated that *wPGRP* is induced by gram-negative bacteria and that the level of induction depends on bacterial growth. Real-time reverse transcription-PCR quantification of the *wPGRP* gene transcript performed at different points in insect development has shown a high steady-state level in the bacteria-bearing organ (the bacteriome) of larvae and a high level of *wPGRP* up-regulation in the symbiotic nymphal phase. Concomitantly, during this stage fluorescence in situ hybridization has revealed an endosymbiont release from the host bacteriocytes. Together with the previously described high induction level of endosymbiont virulence genes at the nymphal phase (C. Dale, G. R. Plague, B. Wang, H. Ochman, and N. A. Moran, *Proc. Natl. Acad. Sci. USA* 99:12397–12402, 2002), these findings indicate that insect mutualistic relationships evolve through an interplay between bacterial virulence and host immune defense and that the host immunity engages the *PGRP* gene family in that interplay.

Among the striking attributes of eukaryotic organisms is their ability to evolve close associations with diverse bacteria, with a continuum of phenotypes ranging from pathogenic to mutualistic (28, 29). However, the molecular mechanisms and responses of the host immune system underlying a given phenotype remain poorly understood, particularly in invertebrate intracellular symbiosis. In insects, endosymbionts are maternally inherited, and early insect embryogenesis is marked by bacterial invasion and the subsequent differentiation of specialized host cells, called bacteriocytes, that often form an organ called the bacteriome (4, 18). Functions of the bacteriocyte cell include nutrient synthesis, molecular exchange between the host and the bacteria (12, 21), and presumably the control of endosymbionts and their isolation from the insect immune system.

Insect intracellular symbiosis increases host fitness and invasive power (17) and, over evolutionary time, leads to a complete interdependence between the bacteria and the host. Due to evolutionary constraints associated with a strictly intracellular life style and maternal inheritance of the symbionts, selection pressure favors, during the host-symbiont coevolution, deletions of bacterial genes encoding redundant metabolic pathways with the host (34), DNA recombinational repair genes (11), and genes becoming “unnecessary” to the new

association. Among the latter, virulence genes and genes encoding the bacterial cell wall elements have been prone to serial gene deletions, as evidenced in insect endosymbiont genomes sequenced so far (1, 15, 34). Hence, association features may rely not only on the host functions but also on the level of bacterial genomic alteration and, thereby, on the age of the association. More recent associations have fewer bacterial genomic alterations than their free-living relatives. In this evolutionary context, recent associations with less deleted bacterial genomes should provide insight into the early stages in the interplay between host immunity and bacterial virulence. Among insect endosymbioses, the *Sitophilus* species associations provide an excellent model for studying innate immune responses, because the association was established relatively recently (less than 25 million years ago), probably by endosymbiont replacement (26). The endosymbiont genomes have not experienced severe gene deletion (7), and these genomes encode functional secretion systems (10).

Recent studies have uncovered a remarkable conservation in innate immune defense mechanisms among plants, insects, and mammals, which suggests a common ancestry of the system (6, 23). An initial step of the immune reaction is the perception of conserved microbial cell envelope motifs, such as peptidoglycan (PGN), through recognition receptors (22). Among these are the peptidoglycan recognition proteins (PGRP) that recognize the DAP type and/or the Lys type of PGN from gram-negative and gram-positive bacterial peptidoglycan, respectively (25, 27). These evolutionarily conserved proteins contain a domain with homology to the bacteriolytic enzyme lysozyme, but some have lost the corresponding amidase activity (30).

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Moreover, the PGRP family members exhibit variability in protein localization and function. In *Drosophila melanogaster*, the *PGRP-SA* and *PGRP-SD* genes are up-regulated upon infection, and they encode secreted proteins that mediate the response to gram-positive bacteria through the activation of the Toll pathway, which leads to antimicrobial peptide synthesis (2, 31). In contrast, the *PGRP-LC* gene is constitutively expressed and has been shown to be a receptor of the Imd pathway, which is activated in response to gram-negative bacterial infection (8, 9, 16). Recently, however, other PGRP family members, namely *PGRP-SC1/2* and *PGRP-LB*, have been shown to reduce the immunostimulatory potency of PGN through an amidase activity, which downregulates the Imd pathway after bacterial challenge (3, 24, 30, 35, 36). Therefore, because of their diversity, *PGRP* genes may be responsible for a large spectrum of modulated immune responses to commensal mutualistic and pathogenic microorganisms.

In the present work, we have compared the expression of a weevil *Sitophilus zeamais* *PGRP* gene (*wPGRP*) ortholog to that of *Drosophila* *PGRP-LB* in response to the intracellular symbiont SZPE (*Sitophilus zeamais* primary endosymbiont). We show for the first time that *PGRP* gene transcripts are accumulated in the presence of endosymbiotic bacteria in the bacteriome organ. A real-time reverse transcription-PCR (RT-PCR) transcript survey at different points of insect development has revealed a high level of *wPGRP* gene induction in the symbiotic nymph, the unique phase where SZPE virulence genes were previously shown to be induced (10). Fluorescence in situ hybridization (FISH) endosymbiont monitoring has demonstrated that *wPGRP* gene induction is concomitant with endosymbiont release from the bacteriocytes. These findings show that the *PGRP* gene family is involved in host-symbiont interaction and indicate that cooperative relationships evolve from the interplay between bacterial virulence and host immune defense.

MATERIALS AND METHODS

Insect rearing and strain selection. Insects were reared on wheat grains at 27.5°C and 70% relative humidity. Many *S. zeamais* weevils are naturally infected with two intracellular bacteria: the bacteriocyte-inducing γ -proteobacterium, SZPE, and an α -proteobacterium *Wolbachia* sp. that interferes with insect reproduction (18). To obtain a one-host/one-symbiont model, and to avoid any *Wolbachia* side effects on the host gene expression, an SZPE-monosymbiotic strain (*S. zeamais* fagoa) was selected and used in this work. Moreover, aposymbiotic (artificially nonsymbiotic) insects were also obtained as described elsewhere (33). The aposymbiotic status is checked by larval dissection (absence of bacteriome) and by PCR using SZPE-specific primers.

Weevil larvae and nymphs normally grow inside the wheat grains until they emerge as adults 1 month after the egg laying. In this work, fourth-instar larvae were dissected from the grains and kept alive in a moist atmosphere at 27.5°C for the length of the experiment.

Bacterial challenge and bacterial growth monitoring. Fourth-instar aposymbiotic larvae were challenged by pricking with sterile sharpened needles (mock infection) or with needles previously dipped into a bacterial solution. Either exponential-phase cultures (optical density at 600 nm, 0.8) of *Pseudomonas aeruginosa* (PAO1 strain) or pellets from overnight cultures for *Escherichia coli* (TOP10; Invitrogen) were used to infect the aposymbiotic larvae. An exponential-phase culture of *E. coli* was used at first but has failed to induce a significant infection (data not shown). For Northern blot experiments and bacterial growth monitoring, groups of 20 larvae were pricked and kept in a moist atmosphere at 27.5°C for 1, 2, 6, 12, and 24 h. Dead larvae were counted, and living individuals were either stored at -80°C for RNA preparation or treated for bacterial growth assays. Naive aposymbiotic larvae were kept in the moist atmosphere and analyzed in parallel as controls.

Bacterial growth was determined by the number of bacteria cells present in groups of five aposymbiotic infected larvae homogenized in 200 μ l of Luria broth (LB). The homogenate was centrifuged for 10 min at 300 $\times$ g to remove larval debris, and the supernatant was serially diluted and spread on LB agar plates. Naive aposymbiotic larvae were used in parallel as controls.

Northern blot experiments. Northern blot experiments were performed on total RNA extracted from insect tissues (triplicate groups of 10 larvae) by the TRIzol Reagent (Invitrogen), following the manufacturer's instructions. For each sample, approximately 12 μ g of total RNA was run on the gel as described previously (20). RNA was transferred overnight onto a Hybond N membrane (Amersham), and membranes were baked for 2 h at 80°C. Membranes were prehybridized, hybridized, and washed as described previously (18). The *wPGRP* probe was a 533-bp fragment (GenBank accession no. CN612423), cloned in pCR2.1-Topo (Invitrogen), obtained from a subtractive cDNA library that had been created previously for weevil's bacteriocytes (21). The total amount of RNA loaded was normalized by hybridization of the blots with a 744-bp fragment of β -actin amplified by PCR using forward primer (For) 5'-AGATGACMCAGATCATGTTTCG-3' and reverse primer (Rev) 5'-CCRGACTCGTCRTACTCTCTG-3' and the weevil's DNA as the template. Probes were labeled with [α -³²P]dCTP (10 mCi/ml) by random priming using the prime-a-gene labeling system kit (Promega) and purified using the Quick-Spin columns (Boehringer, Mannheim). Blots were exposed for up to 24 h to a Storm PhosphorImager imaging plate (Amersham), and the band intensities were quantified using the ImageQuant software. Normalized data were analyzed using the analysis of variance (ANOVA) test.

Real-time RT-PCR transcript quantification. Real-time RT-PCR transcript quantification was performed in experiments involving small amounts of tissue material (i.e., bacteriomes, oocytes, or embryos). Three independent RNA extractions were made on each sample. RNA was incubated with 1 U/ μ g of RQ1 RNase-Free DNase (Promega) for 30 min at 37°C, followed by enzyme inactivation with 1 μ l of the stop solution (Promega) at 65°C for 10 min. After purification with the RNeasy mini kit (QIAGEN), RNA was quantified with the Ribogreen RNA quantification kit (Molecular Probes) and stored at -80°C for future use. Reverse transcription into the first-strand cDNA was made with the First Strand Synthesis System for RT-PCR kit (Invitrogen). The quantification was performed with a LightCycler instrument using the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics). Data were normalized using the ratio of the target cDNA concentration to that of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. The *GAPDH* gene was used as the control in this experiment, because the β -actin gene is repressed in the bacteriome tissue (21). The following primers were designed to amplify fragments with less than 300 bp: For, 5'-ATAATTCGCTGTTGGAGGG-3', and Rev, 5'-TC TCGGACTTGCTATGACC-3', for *wPGRP* (248 bp); For, 5'-AACTTTGCC GACAGCCTGG-3', and Rev, 5'-GCGCCCATGTATGTAGTTGG-3', for *GAPDH* (277 bp).

The PCRs were carried out in LightCycler capillaries in a final volume of 20 μ l containing 2 μ l of cDNA samples (diluted 10-fold), 4 mM MgCl₂ (3.5 mM for *GAPDH*), 0.5 μ M of each primer, and 2 μ l of LC-Fast Start Reaction Master SYBR Green I. After 8 min at 95°C, the cycling conditions were as follows: 45 cycles at 95°C for 10 s, 58°C for 6 s for *wPGRP* (or 62°C for 8 s for *GAPDH*), and 72°C for 12 s (16 s for *GAPDH*). For product identification, a melting curve was constructed at the end of each PCR by heating for 15 s at 68°C (72°C for *GAPDH*) and increasing the temperature up to 95°C, with increment rates of 0.1°C/s. Reactions were achieved by cooling at 40°C for 30 s.

The PCR efficiency (96% for *GAPDH* and 92% for *wPGRP* in this study) and, for the individual samples, the crossing point and the concentration (conc.) of the *wPGRP* (or the *GAPDH*) transcripts were determined. As the quantification relies on the PCR efficiency of each experiment, ratios were normalized with the ratio of a relative standard (the calibrator), which is a sample prepared from symbiotic larval RNA and used in each assay. The relative ratio for each sample was calculated according to the following formula: [conc. *wPGRP* (sample)/conc. *GAPDH* (sample)]/[conc. *wPGRP* (calibrator)/conc. *GAPDH* (calibrator)]. Normalized data were analyzed using the ANOVA test.

Fluorescence in situ hybridization (FISH). The presence of SZPE was monitored by FISH experiments from the time of embryo hatching until the adult stage. Synchronously developing weevils were obtained by allowing 2- to 4-week-old insects to lay their eggs for 2- to 4-h periods either on starch pellets (for embryos and first-instar larvae) or on wheat grains (for nymphs and adults). Embryos and larvae were collected by dissolving the starch with water, and nymphs were collected from the grains. Tissues were fixed, embedded in paraffin, cut, and mounted on poly(L-lysine)-coated microscope slides as described previously (18). After methylcyclohexane dewaxing and rehydration, sections were covered by a drop of 70% acetic acid and stored at 45°C until the drop had dried.

FIG. 1. Bacterial growth in aposymbiotic infected larvae. Larvae were infected with either *E. coli* or *P. aeruginosa*. The number of bacteria was measured at 2, 6, 12, and 24 h after infection. Data are expressed as CFU (CFU per larva) and represent the means of two samples of five larvae.

Sections were then dehydrated, prehybridized, hybridized with an SZPE-specific 5'-end rhodamine-labeled 16S rRNA gene probe (5'-ACCCCCCTCTACGAG AC-3'), washed, and mounted in DAKO Fluorescent Mounting Fluid containing 6 μ g/ml of 4',6-diamidino-2-phenylindole (DAPI), as described previously (18).

RESULTS

Larval bacterial infection and *wPGRP* gene expression. To test whether the *wPGRP* gene is regulated in response to gram-negative bacteria, we infected aposymbiotic larvae and quantified *wPGRP* transcript steady-state levels. To determine how *wPGRP* responds both to nonpathogenic and pathogenic gram-negative bacteria, larvae were challenged with *Escherichia coli*, which is quickly eliminated by the insect immune system, and with *Pseudomonas aeruginosa*, which proliferates in insects and kills them (14). Under these conditions, *wPGRP* gene expression could be monitored in relation to bacterial growth and virulence status.

Bacterial infection and growth. To estimate the susceptibility of the larvae to infection by *E. coli* or *P. aeruginosa*, the rate of proliferation of these bacteria was estimated by monitoring bacterial plate counts, expressed as CFU recovered from aposymbiotic infected larvae at different times after infection (Fig. 1). As expected, no CFU were recovered on LB plates from the unchallenged aposymbiotic larvae (data not shown). Two hours following infection, the CFU per larva was higher for *E. coli* than for *P. aeruginosa* (50-fold), which reflects the initial concentration of the bacterial solution used for infection (see Materials and Methods). Subsequently, no apparent change in the number of *E. coli* was seen from 2 to 12 h, at which time a slight decrease was observed. In contrast, the pathogenic *P. aeruginosa* rapidly proliferated, reaching 1.5×10^6 CFU/larvae.

Intriguingly, analysis of larval survival showed that during the 24-h challenge, infection by either *P. aeruginosa* or *E. coli* did not affect larval viability compared with the viability of the mock-infected larvae (data not shown). The larval viability cannot be analyzed after 24 h postinfection, because larvae dissected from the wheat grains do not feed, which impairs their physiology and their survival.

FIG. 2. Quantification of *wPGRP* gene transcripts from control and challenged aposymbiotic larvae. (A) Shown are the images of the Northern blot hybridized with *wPGRP* and with β -actin probes. Cal, calibrator sample; C, unchallenged aposymbiotic larvae; S, mock-infected larvae; E and P are larvae infected with *E. coli* and *P. aeruginosa*, respectively; numbers 1, 2, and 3 indicate sample repetitions. Larvae were incubated for 1, 2, 6, and 12 h following the treatment. (B) Levels of *wPGRP* gene transcripts normalized with the expression of the β -actin gene. Data (expressed in arbitrary units) are means of three independent repetitions normalized with the relative intensity of the β -actin gene. Bars represent the standard deviations. Data were analyzed with ANOVA (see Results).

***wPGRP* gene expression.** Northern blot experiments were conducted on RNA isolated from aposymbiotic larvae after mock infection or an infection with *E. coli* or *P. aeruginosa* as well as from untreated aposymbiotic larvae (Fig. 2A). The results showed that the *wPGRP* gene was expressed at a basal level in the naive larvae, which suggests a constitutive expression by insect immunocompetent tissues. The level of transcripts changed according to the experimental conditions (Fig. 2B).

No difference was seen between the controls and the challenged larvae 1 h postinfection ($P < 0.80$), and the *wPGRP* transcript level did not change significantly with the postinfection time in control larvae ($P < 0.13$) or after mock infection ($P < 0.15$). Moreover, compared with control larvae, the level

FIG. 3. Real-time RT-PCR quantification of *wPGRP* gene steady-state levels at different points of the weevil life cycle. Total RNA was extracted from bacteriomes (B), oocytes (O), 3-day-old embryos (E), fourth-instar larvae (L), nymphs (N), and 3-week-old adults (A) of symbiotic (black) and aposymbiotic (gray) weevils. Each bar represents the means of three independent measurements with standard deviations after normalization.

of *wPGRP* transcripts was not significantly increased by the mock infection ($P < 0.053$).

In contrast, *wPGRP* mRNA levels were shown to increase up to 5.5-fold with *E. coli* and up to 8.5-fold with *P. aeruginosa* (Fig. 2B). Using 1 h postinfection as a reference, the effect of bacterial infection on *wPGRP* gene expression was significant at 2 h and at 6 h postinfection for *E. coli* and at 2 h and at 12 h postinfection for *P. aeruginosa*.

***wPGRP* gene expression at different points in the host life cycle: comparison between symbiotic and aposymbiotic insects.** To obtain insights into the host immune response to SZPE in the bacteriome tissue and according to the evolution

of symbiotic structures during host development, we have determined the amount of *wPGRP* transcript present in the bacteriome of the fourth-instar larvae and in the whole insects at different developmental stages (i.e., oocytes, embryos, fourth-instar larvae, nymphs, and adults). The levels of transcript were obtained by real-time RT-PCR, using the *GAPDH* gene as an internal control.

The expression of the *wPGRP* gene in the bacteriome samples was high, confirming the previous data obtained using a cDNA subtraction approach (21). Figure 3 shows that the *wPGRP* transcript level is 160-fold higher in the bacteriome than in the whole aposymbiotic larvae.

The value of the *wPGRP* transcript level changed during the development of both aposymbiotic ($P < 5 \times 10^{-6}$) and symbiotic individuals ($P < 3.64 \times 10^{-7}$). Indeed, while the amount of *wPGRP* transcript was high in the oocytes (regardless of symbiotic status), it decreased significantly during the first three days of embryogenesis (Fig. 3). The nymphal stage was the unique point in the host life cycle (among those tested in this experiment) that exhibited a significant difference with respect to symbiont presence or absence ($P < 2.12 \times 10^{-5}$). For instance, despite the high level of *wPGRP* transcripts in the larval bacteriome, no significant difference was seen between whole symbiotic and aposymbiotic insects at this stage. This is likely due to the very small representation of the bacteriome tissue within the insect body as well as to the "dilution" of the bacteriome *wPGRP* transcripts by the basal level of gene expression in insect tissues (see values from aposymbiotic insects). In the nymphal stage, however, the amount of *wPGRP* transcripts was 10-fold higher in symbiotic than in aposymbiotic nymphs (Fig. 3). This is most likely the result of *wPGRP* gene induction (either within the bacteriocytes or in

FIG. 4. FISH monitoring of SZPE at different phases of insect development. Shown are DAPI visualizations of whole embryos from *S. zeamais* at (image 1) 30 min, (image 2) 6 h, (image 3) 3 days, and (image 4) 4 days, respectively. Images 5, 6, and 7 are SZPE magnifications from the areas indicated by the arrows in images 1, 2, and 3, respectively. Images 8 to 13 are rhodamine visualizations of SZPEs in the larval bacteriome intimately attached to the gut (image 8), during the bacteriome dissociation at the first nymphal stages (images 9, 10, and 11), and in the mesenteric caeca bacteriomes of aged nymphs (image 12) and young adults (image 13). AP, anterior pole; PP, posterior pole; hc, head capsule; Lb, larval bacteriome; bm, bacterial migration; ba, bacterial aggregation; g, gut; mb, mesenteric bacteriome; b, bacteriocyte; gl, gut lumen; at, adipocyte tissue. Arrows indicate the position of SZPEs. Scale bar, 10 μ m.

nymphal immunocompetent tissues), as the bacteriome does not increase in size between the fourth-instar larva and the nymph (19).

SZPE behavior at different phases of insect development: endosymbiont externalization at the nymphal phase. FISH experiments were used to monitor SZPE presence and behavior throughout weevil development. At least five independent individuals were analyzed for each phase. SZPEs were concentrated at the posterior pole of oocytes and young embryos (Fig. 4, images 1 and 5) in close association with the germ cells. Embryo cleavage begins 6 to 7 h after the egg laying (Fig. 4, image 2).

As cleavage progresses, the SZPE begins to move out to the central part of the embryo (Fig. 4, image 6), where a large previously unreported bacterial mass aggregates on the third day, just before entering the bacteriocytes (Fig. 4, images 3 and 7). On the fourth day, the bacteriome organ is well structured and intimately attached to the intestine (Fig. 4, image 8). From this phase until the end of the fourth-instar larval stage, a careful FISH screening throughout the whole body failed to reveal any SZPE cell outside the bacteriocytes. However, as nymphal metamorphosis begins, the bacteriome dissociates and many bacteria are found outside the bacteriocyte cells (Fig. 4, images 9 and 10).

Interestingly, SZPE seems to proliferate only in the gut lumen (Fig. 4, images 9, 10, and 11), while it fails to invade the adipocyte tissue (Fig. 4, image 11). In the longer term, many bacteriocytes are aligned as a single cellular layer along the intestine, and endosymbionts are no longer extracellular (Fig. 4, image 11). Bacteriocytes group together in old nymphs and young adults to form the bacteriome of the mesenteric caeca (Fig. 4, images 12 and 13).

DISCUSSION

A permanent association between hosts and bacteria implies the existence of control mechanisms that regulate the symbiont population to avoid uncontrolled proliferation and subsequent host damage by the bacteria and, conversely, to support the maintenance of the bacteria at a certain level, presumably by providing nutrients and modulating immunity. Since the work of Buchner (5), insect intracellular symbiosis has been extensively described, but it has generally been described without regard to the interplay between the insect innate immune system and bacterial virulence. Nevertheless, the restricted area of symbiont growth within the bacteriocytes raises the question of whether, and how, these specific cells perceive, control, and maintain the bacteria.

Recently, we have studied *S. zeamais* symbiosis, and we have discovered an intriguing bacteriocyte immune response to the endosymbiont: the expression of a member of the *PGRP* gene family (21). This family includes inducible and constitutive proteins that are involved either in activation or down-regulation of the host immune response (3, 13, 36). In the context of weevil symbiosis, endosymbiont control may rely upon a regulated system that senses bacterial cell fluctuation and physiological status during insect development. To characterize *wPGRP* with respect to gene regulation, we have measured its expression in response to *P. aeruginosa* and *E. coli* bacterial challenge of aposymbiotic naive larvae. Northern blot analysis

showed clearly that the *wPGRP* transcript steady-state level is greatly increased after a bacterial challenge (Fig. 2), thus demonstrating that nonsymbiont gram-negative bacteria can induce *wPGRP* gene expression. Interestingly, this expression did not show identical profiles with *E. coli* and with *P. aeruginosa* (Fig. 2), and *wPGRP* transcript accumulation was shown to parallel bacterial growth in the aposymbiotic-infected larvae (Fig. 1). These findings strongly support that *wPGRP* gene expression depends on bacterial growth, which assigns to *wPGRP* the status of an immune gene suitable for the host perception of endosymbiont cell density and growth.

Among the attributes of endosymbionts in the insect world is the absence of virulence and tissue invasion. However, Dale et al. (10) have recently shown in *S. zeamais* that the endosymbiont SZPE expresses, during the host nymphal phase, *inv/spa* genes that are involved in the type three secretion system associated with cell invasion. The reason for this remains unclear, but it may be related to physiological and hormonal changes that occur during this phase of metamorphosis. One possibility is that endosymbiont *inv/spa* gene up-regulation is the consequence of bacterial release from the bacteriocytes and the subsequent invasion of the surrounding cells. To explore this hypothesis, and to test whether the *wPGRP* gene expression evolves according to the change in the endosymbiont physiological status, we have conducted FISH experiments to monitor SZPE location in parallel with a real-time RT-PCR *wPGRP* transcript survey at different stages in the host life cycle.

Two striking observations were made: (i) a *wPGRP* transcript accumulation within the bacteriome, and (ii) concomitant *wPGRP* gene induction and SZPE release from the bacteriocytes in the nymphal phase.

(i) This is the first time that we have been able to demonstrate an up-regulation of an immune defense gene in insect bacterial intracellular symbiosis. *wPGRP* transcript accumulation in the bacteriome is interpreted as being a response of the insect immune system to the symbiont.

However, the function of the *wPGRP* gene remains speculative and needs more investigation for us to understand whether this gene triggers antibacterial peptide synthesis via an Imd-like pathway or whether it possesses amidase activity and, at the opposite end, whether it downregulates an Imd-like pathway. It is likely that the *wPGRP* protein possesses amidase activity, since the five amino acid residues required for this enzymatic activity (30) are well conserved in this weevil gene (21). In this case, the overexpression of the *wPGRP* gene in the bacteriocytes would prevent activation of an Imd-like pathway and therefore would help symbiont persistence in these symbiont-bearing cells. This hypothesis is strongly supported by the function of the *wPGRP* ortholog in *Drosophila* (i.e., *PGRP-LB*) that is transcribed mostly in the larval gut as *PGRP-SC1* and *PGRP-SC2* genes, probably to prevent a permanent activation of the Imd pathway in response to gut microbial flora (3, 36).

(ii) Insect primary endosymbionts have always been described as being intracellular during the insect life cycle. In *Sitophilus* spp., several authors have described the bacteriome dissociation during nymphosis and the bacteriocyte "migration" along the intestine (19). However, neither these authors nor those of other studies have firmly demonstrated whether

SZPE exits from the bacteriocytes during this phase. The observation that SZPE increases up to 20-fold the expression of *inv/spa* virulence genes during metamorphosis (10) raises the question of whether the change in SZPE transcription parallels bacterial release and whether, and then how, the weevil immune system responds to this symbiont physiological change.

In this work, FISH experiments have shown that SZPE remains intracellular until the last larval stages (Fig. 4, image 8). However, as soon as metamorphosis begins in the pre-nymph stage the larval bacteriome dissociates, and numerous bacteria were shown outside the bacteriocytes (Fig. 4, image 9). Bacterial growth and invasion are limited at the intestine side exposed to the adipocyte tissue, probably due to the immune activity of this tissue, while many bacteria are found in the gut lumen (Fig. 4, images 10 and 11). This finding calls into question the formation of the adult midgut caeca bacteriomes that may result from cell reinfection along the intestine, in addition to (or instead of) the bacteriocyte migration described previously (19).

In parallel to bacterial cell release in the nymphal phase, a high level of *wPGRP* gene up-regulation was shown (Fig. 3). This gene up-regulation, along with bacterial *inv/spa* gene induction (10), indicates that host-symbiont interaction involves the interplay between host immune systems and bacterial virulence genes. However, the function of both immune and virulence genes needs to be determined in the context of mutualistic associations. Whatever the role of the *wPGRP* gene (activator or downregulator), its induction could be regarded as an adaptive immune response to bacterial release in order to prevent bacterial invasion into insect tissues and complete bacterial clearance. The relatively high prevalence of SZPE in the digestive tract, and its absence from the adipocyte tissue, are in line with this assumption and suggest, instead, bacterial elimination from and by this immunocompetent tissue.

In conclusion, the study described here shows for the first time that the *PGRP* gene family is prone to interact with insect endosymbiosis. The *wPGRP* gene is regulated by gram-negative bacteria, its expression changes according to bacterial growth, it is up-regulated in the bacteriocytes, and it responds actively to SZPE release from the bacteriocytes and/or to the change in SZPE gene transcription. The future investigation of the molecular mechanisms of *wPGRP* regulation and activity (activator or down-regulator) and of *wPGRP*-SZPE interaction will contribute to the understanding of how prokaryotes and eukaryotes interact to favor mutualism at the initial step of symbiosis. So far, no data have reported innate immune genes in those associations, except the recent up-regulation of an i-type lysozyme gene recently described in the aphid bacteriocytes (32).

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