Systemic Infection Generates a Local-Like Immune Response of the Bacteriome Organ in Insect Symbiosis

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Key Words

Bacteriome · Symbiosis · Immune response · Insect ·
Sitophilus weevils · Endosymbiont · *Dickeya* ·
Antimicrobial peptide

Abstract

Endosymbiosis is common in insects thriving in nutritionally unbalanced habitats. The cereal weevil, *Sitophilus oryzae*, houses *Sodalis pierantonius*, a Gram-negative intracellular symbiotic bacterium (endosymbiont), within a dedicated organ called a bacteriome. Recent data have shown that the bacteriome expresses certain immune genes that result in local symbiont tolerance and control. Here, we address the question of whether and how the bacteriome responds to insect infections involving exogenous bacteria. We have established an infection model by challenging weevil larvae with the Gram-negative bacterium *Dickeya dadantii*. We showed that *D. dadantii* infects host tissues and triggers a systemic immune response. Gene transcript analysis indicated that the bacteriome is also immune responsive, but it expresses immune effector genes to a lesser extent than the systemic and intestinal responses. Most genes putatively involved in immune pathways remain weakly expressed in the bacteriome following *D. dadantii* infection. Moreover, quantitative PCR experiments showed that the endosymbiont load is not affected by insect infection or the resulting bacte-

riome immune activation. Thus, the contained immune effector gene expression in the bacteriome may prevent potentially harmful effects of the immune response on endosymbionts, whilst efficiently protecting them from bacterial intruders.

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Introduction

Insects thriving in a nutritionally unbalanced environment often evolve a mutualistic relationship with symbiotic intracellular bacteria (endosymbionts). Endosymbionts complement the insect's diet with metabolic components and contribute considerably to its adaptability and invasive power [1, 2]. A symbiotic relationship such as this can be seen in the association of the cereal weevil (*Sitophilus oryzae*) with the Gram-negative γ -proteobacterium *Sodalis pierantonius* [3–6]. Weevil endosymbionts are transmitted maternally to the insect progeny. At early stages of embryonic development, insects differentiate specialized cells, called bacteriocytes, which group together to form the bacteriome organ in the larva. The bacteriocytes house endosymbionts and isolate them from the host systemic immune response [7, 8]. Endosymbionts are only tolerated inside these specific cells and their externalization from the bacteriocytes results in

the activation of a systemic response and the secretion of antimicrobial peptides (AMP) [6]. This suggests that efficient mechanisms have been selected through host-symbiont coevolution that allow bacteriocytes to both protect and maintain the symbionts, and to control their localization, growth and density [9, 10]. In endosymbiotic models, the bacteriome organ exhibits a specific immune transcriptional profile when compared with other tissues [7, 11–14]. In *S. oryzae*, this includes the intracellular expression of the AMP coleopteracin A, which specifically targets endosymbionts and inhibits their cell division through interaction with the chaperonin GroEL [9]. However, the bacteriome immune functions might not be limited to endosymbiont control and they may also help to protect this symbiont-bearing organ from opportunistic pathogen infections that could damage the bacteriocytes and their associated endosymbionts.

To tackle this question, we have established an infection model in *S. oryzae* weevils by challenging the larvae with the Gram-negative bacteria *Dickeya dadantii*. The humoral immunity of insects to bacterial infections is best described in *Drosophila melanogaster*, where the recognition of bacterial peptidoglycan by pattern recognition receptors (such as the peptidoglycan recognition proteins; PGRP) selectively activates the Toll and immune deficiency (IMD) signaling cascades [15]. Both pathways lead to the activation of transcription factors of the NF- κ B family and the transcriptional induction of various immune effector genes, such as AMP coding genes. The systemic response relies on the activation of Toll or IMD pathways in the fat body and a massive secretion of AMP into the insect hemolymph, where they act against bacterial intruders. The local immune responses rely on the activation of the IMD pathway in epithelia that are in contact with bacterial intruders, and which generate a local antimicrobial activity. In this study, tissue-specific analysis of the expression of immune-related genes showed that *D. dadantii* injected into the weevil hemolymph triggers a strong systemic immune response and results in a low but significant response from both the midgut and the bacteriome. Although similar to the local intestinal response, the bacteriome response clearly presented some qualitative and quantitative specificity and appeared to be more contained. Importantly, the endosymbiont load was not affected by insect infections. These results strongly suggest the existence of an immunomodulation process that maintains the bacteriome immunity at a low level of activation, presumably to avoid collateral damage to the endosymbionts and to limit symbiont exposure to bacterial intruders.

Materials and Methods

Insect Rearing, Infection and Sample Preparation

S. oryzae weevils were reared on wheat grains at 27.5°C and at 70% relative humidity. The Bouriz strain of *S. oryzae* was chosen because it is free of any facultative symbionts, such as *Wolbachia*, and it harbors only *S. pierantonius*. Aposymbiotic insects were obtained as previously described [16]. Septic injuries were made on fourth instar larvae challenged with 10^5 bacteria of the genus *Micrococcus luteus* (strain CCM169) or *D. dadantii* (strain A470). A total of 69 nl of bacterial suspension were injected into the hemolymph using a Nanoject II (Drummond).

For convenience, and due to the small larval body size, the whole larva was used to estimate the systemic response since the epithelial immune responses are negligible compared to the strong systemic response generated by the fat body. Aposymbiotic insects were used in this assay, taking advantage of the fact that they are devoid of bacteriomes.

For organ-specific studies, bacteriomes and guts were dissected from fourth instar symbiotic larvae in Buffer A (25 mM KCl, 10 mM MgCl₂, 250 mM sucrose, 35 mM Tris/HCl, pH = 7.5). For each sample, at least 4 whole larvae or 20 organs were pooled and stored at –80°C prior to RNA extraction. To take into account any individual variability, each sampling was independently repeated three times for the end-point reverse transcription quantitative PCR (RT-qPCR), four separate times for the kinetics analysis and five times for the bacterial counts by qPCR.

Identification of Genes of Interest

Cecropin, *coleopteracin A* and *B*, *dipteracin*, *fk506bp*, *GGBP1*, *imd*, *sarcotoxin*, *tollIP*, *wPGRP1*, 2 and 3 were all identified from previous publications [7, 12, 13]. *Caudal*, *pirk*, *defensin*, *relish* and *toll* were identified using unpublished RNAseq data. The respective sequences can be found under the following accession numbers: KM034776, KM034777, KM034778, KM034779 and KM034780.

Total RNA Extraction and Reverse-Transcription

Total RNA from whole larvae was extracted with the Trizol reagent (Invitrogen) following the manufacturer's instructions. RNA was incubated with 1 U/ μ g of RQ1 RNase-free DNase for 30 min at 37°C. The total RNA of bacteriomes and guts 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 (Thermo Scientific) and RNA quality was checked using agarose gel electrophoresis. Reverse-transcription into the first strand cDNA was carried out using the First Strand Synthesis System for RT-PCR kit (Invitrogen).

Real-Time RT-qPCR Transcript Quantification

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 two house-keeper genes: *glyceraldehyde 3-phosphate dehydrogenase (gapdh)* and *ribosomal protein L29 (rpL29)*. The expression of these genes is not significantly influenced by the treatments employed. Primers were designed to amplify fragments of approximately 200 bp. A complete list of the primers can be found in online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000368928).

The PCR reactions were carried out in LightCycler 96-well plates in a final volume of 10 µl, containing 2.5 µl of cDNA samples (diluted 5-fold) and 7.5 µl of LightCycler 480 SYBR Green Master 1 mix, with 0.5 µl of 10 mM of each primer, 1.5 µl H₂O and 5 µl of Mastermix. After 5 min at 95°C, the cycling conditions were as follows: 45 cycles at 95°C for 10 s, 56°C for 20 s and 72°C for 30 s. For product identification, a melting curve was constructed at the end of each PCR by heating for 30 s at 66°C and then increasing the temperature up to 95°C with increment rates of 0.11°C/s. Reactions were terminated by cooling at 40°C for 30 s. For each individual sample, the crossing point and the concentration of the gene transcripts were determined. Ratios were then normalized with the *gapdh* and *rpl29* genes.

DNA Extraction and qPCR Bacterial Count

Total DNA was extracted from pools of five larvae by crushing them with an RNase-free pellet pestle and using a NucleoSpin Tissue Kit (Macherey-Nagel). DNA quantification was carried out using the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics). The target was a 200-bp fragment of NADH-ubiquinone oxidoreductase subunit CD (*NuoCD*; the primers used were: 5-CACAGCCAAATGTGGTGAAG-3 and 5-GCAGGTCATAGAGCATCACA-3) for *S. pierantonius* quantification, and indigoidine biosynthesis protein A (*IndA*; the primers used were: 5-TATTGTCGTTCAGCGGTTT-3 and 5-CCCACGAATACCTTCATGCT-3) for *D. dadantii* quantification. The PCR reactions were carried out in LightCycler 96-well plates in a final volume of 10 µl, containing 1 µl of DNA samples and 9 µl of LightCycler 480 SYBR Green Master 1 mix, with 0.5 µl of 10 mM of each primer, 3 µl H₂O and 5 µl of Mastermix. After 10 min at 95°C, the cycling conditions were as follows: 45 cycles at 95°C for 10 s, 58°C for 10 s and 72°C for 10 s. For product identification, a melting curve was constructed at the end of each PCR by heating for 1 min at 68°C, and then increasing the temperature up to 95°C with increment rates of 0.11°C/s. Reactions were terminated by cooling at 40°C for 30 s. For each individual sample, the crossing point and the concentration of the DNA fragment were determined. Bacterial DNA quantifications were normalized using the ratio of the target bacterial gene (*NuoCD* or *IndA*) with that of the host β -actin gene (the primers used for β -actin amplification were: 5-GCCTCAACCTCCCTAGAAAA-3 and 5-GGTGTTGGCGTACAAAGT CCT-3).

Fluorescence in situ Hybridization

D. dadantii localization in insect tissues was analyzed by fluorescence in situ hybridization (FISH) experiments on whole symbiotic larvae at 6, 12 and 24 h after injection of the bacterial suspension or sterile PBS buffer. At the end of the incubation period, larvae were fixed in PBS with 4% paraformaldehyde, embedded in paraffin, cut and then mounted on poly(L-lysine)-coated microscope slides.

After methylcyclohexan dewaxing and rehydration, sections were covered with a drop of 70% acetic acid. The sections were then prehybridized, hybridized with a *D. dadantii*-specific 5'-end TAMRA-labeled oligo-probe targeting 16S RNA (5'-CCC-CGT-ATC-TCT-ACA-GGG-3'), washed and then mounted in Perma-Fluor Mounting Fluid (ThermoScientific) containing 3 µg/ml of 4', 6-diamidino-2-phenylindole (DAPI), as previously described [17].

Images were acquired with an epifluorescence microscope (Olympus IX81 equipped with a HQ535/50 filter for green signal,

D470/40 for blue signal and HQ610/75 for red signal) and captured using an F-ViewII camera and the CellF software (Soft Imaging System). Images were treated and analyzed using ImageJ (release 1.47v).

Statistical Analysis

Transcriptomic data on humoral and bacteriome immune response kinetics were analyzed using linear models on the log-transformed gene expression data. The type of treatment was considered as a 3-level categorical variable representing the injection of sterile PBS (control), *M. luteus* or *D. dadantii*. The time postinfection was considered as a 4-level categorical variable. A two-way analysis of variance (ANOVA) allowing for interaction between the two factors was used to assess the significance of each factor, and the interaction was analyzed using Fisher tests. To refine the analysis, a contrast analysis was run on genes whose expression was significantly affected by the treatment. For models with a significant interaction between the factors 'treatment' and 'time' (interaction p value <0.1), a t test was performed for each given time on the linear model coefficients for pairwise contrasts of each level of the factor 'treatment'. If the interaction was not significant, a new model was built without the interaction calculation, and the contrasts were made globally for the 4 levels of the factor 'time'. The effect of a factor was considered to be significant with a p value <0.05. No adjustments were performed for multiple test comparisons.

Data obtained on the comparison of the bacteriome and the gut immune responses satisfy the requirements for the use of parametrical statistics without any data transformation. The ANOVA and contrast procedure described above have been applied on these data without preliminary manipulation.

All analysis and graphical figures were made using RStudio software v0.98.983 [18] and Hmisc and nlme packages. 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 sample standard deviation and n the sample size.

Results

D. dadantii Infection Triggers an Efficient Systemic Immune Response in Weevil Larvae

To investigate whether the bacteriome generates an immune response to systemic infections with pathogens, we set up an infection model in *S. oryzae* larvae. Insects were infected by an injection of bacteria into the body cavity (hemocoel) using the Gram-negative bacteria *D. dadantii* (formerly *Erwinia chrysanthemi*). *D. dadantii* is known to be virulent for aphids and to have a pathogenic effect when injected into *S. oryzae* larvae [19, 20]. Bacteria injected into insects are known to trigger a systemic immune response generated by their fat body, together with local immune responses from epithelial cells directly exposed to bacteria [15]. However, these two types of response had not been previously assessed in a *Sitophilus/Dickeya* infection model.

The systemic immune response was evaluated by measuring, with RT-qPCR, the transcript levels of six AMP

Fig. 1. Kinetics of AMP gene expression in whole aposymbiotic larvae injected with *M. luteus* or *D. dadantii*. The effect of the type of injection and the effect of time are statistically significant for all

genes. For detailed statistical results, see online supplementary tables 2 and 3. Error bars represent the standard error of four independent measurements.

coding genes: *sarcotoxin*, *coleopteracin A*, *coleopteracin B*, *dipteracin*, *cecropin* and *defensin*. Infections with the Gram-positive bacteria *M. luteus* were carried out in parallel for comparison. To prevent any interference of symbiosis with the infection status, and to avoid any contamination of the systemic immune response by a bacteriome response, we used aposymbiotic insects, which are artificially deprived of endosymbionts [16] and do not grow a proper functional bacteriome. All of the AMP coding genes were strongly activated in whole larvae following these infections. Their expression was slightly higher in response to Gram-negative bacteria compared to Gram-positive bacteria (fig. 1). Except for *coleopteracin B* and *defensin*, which presented stable transcript levels from 12 to 24 h postinfection, all the genes presented similar profiles of expression whereby the highest transcript level was reached 12 h after bacterial injection, followed by a decrease towards 24 h (fig. 1).

The infectious efficacy of *D. dadantii* was monitored by evaluating its ability to grow and spread throughout the insect tissues. Bacterial quantification, using qPCR, showed that the *D. dadantii* population increased from 2 to 12 h after infection, before being eliminated by 24 h (fig. 2a). *D. dadantii* infection of insect tissues was then monitored

using FISH. We looked at the localization of the infectious bacteria through various insect tissues, including the bacteriome. Since aposymbiotic insects do not grow a proper bacteriome, and to take into account the potential interaction between symbiotic and infectious bacteria, we used whole larvae from the symbiotic strain as biological material. At 6 h postinjection, *D. dadantii* could be seen sporadically inside most larval tissues, including the fat body (fig. 2b), muscles (fig. 2c) and the bacteriome organ (fig. 2d). *D. dadantii* was also relatively abundant in the more concealed areas, such as the boundary region of the bacteriome organ or the basal border of the gut epithelium (fig. 2e), where bacteria were sometimes associated with damaged intestinal epithelial cells (fig. 2f). After 12 h, *D. dadantii* was less abundant inside the tissues but, instead, it had accumulated as stacks at the borders of the organs (fig. 2g–i). However, it is worth noting that a strong variability was observed between larvae, depending on how well they had coped with the infection. Healthy-looking larvae housed few *D. dadantii* whereas, in unhealthy larvae with a greenish color, *D. dadantii* had colonized most tissues, including the fat body (fig. 2j), the bacteriome (fig. 2k) and the gut tissues (fig. 2l). By 24 h postinfection, no sign of *D. dadantii* was detected in the surviving larvae.

Fig. 2. Tissue distribution of *D. dadantii* in infected symbiotic larvae. **a** Kinetics of *D. dadantii* counts in whole infected larvae. Bacteria count increases from 2 to 12 h after the injection. At 24 h postinjection, the count falls under the detection limit, indicating bacterial clearance in surviving larvae. Error bars represent the standard error of five independent measurements. At 6 h postinfection, *D. dadantii* could be seen in most tissues of symbiotic larvae, including the fat body (**b**), muscles (**c**) and bacteriome (**d**). It

had also accumulated along the gut epithelium (**e**), sometimes causing damage (**f**). Bacteria were, however, never found inside the host cells. At 12 h postinfection, *D. dadantii* was found mostly on the borders of the organs (**g**). In surviving larvae, bacteria were seen as small stacks along the bacteriome (**h**) and the gut epithelium (**i**) only. In larvae dying from infection, the bacteria had spread to all the tissues, including the fat body (**j**), the bacteriome (**k**) and the gut (**l**).

Fig. 3. Bacteriome immune response in symbiotic larvae injected with *M. luteus* or *D. dadantii*. AMP gene expression was quantified at 2, 6, 12 and 24 h after the bacterial challenge. The effect of the type of injection, the effect of time and their interaction are statis-

tically significant for all genes. For detailed statistical results, see online supplementary tables 2 and 3. Error bars represent the standard error of four independent measurements.

In summary, *D. dadantii* was able to infect *S. oryzae* larvae, to spread inside the organism and to trigger a strong systemic response of the larvae. Survival assays showed that about 50% of the larvae died within 24 h following the infection (see online suppl. fig. 1), while in surviving larvae no infectious bacteria were detected at this time (fig. 2a). Thus, we assumed that all infected larvae that were able to survive had successfully eradicated the infection by 24 h, while others died either from the infection itself or from the strong immune response they triggered.

The Bacteriome Generates a Mild but Effective Immune Response following Bacterial Infection

To determine whether and how the bacteriome responds to a larval bacterial challenge, we infected a weevil symbiotic strain with *D. dadantii*. The AMP gene expression was measured in the dissected bacteriomes. *Dipterucin*, *sarcotoxin*, *cecropin*, *defensin* and *coleoptericin A* and *B* genes were all induced in the bacteriome after infection with either *D. dadantii* or *M. luteus*, as compared with mock-infection controls (fig. 3). The kinetics of these AMP gene expressions in the bacteriome mimic the profiles obtained of the systemic response generated by the

fat body (compare fig. 1 with fig. 3). However, normalized transcript levels of the AMP genes show a higher induction in the systemic response than in the bacteriome. At 12 h after injection, the mean fold-change for the six AMP genes, between *D. dadantii*-infected and mock-infected larvae, was 42-fold for the systemic response and only 14.5-fold for the bacteriome response.

To elucidate the reasons for these differences, we measured the expression profile of genes homologous to key components of *D. melanogaster* antimicrobial NF- κ B pathways, i.e., the Toll pathway (GNBP1, *wPGRP3* and *toll*) and the IMD pathway (*wPGRP2*, *imd* and *relish*), together with putative regulators of these pathways (*wPGRP1*, *caudal*, *pirk*, *fk506bp* and *tollip*; see fig. 4 for expression profiles and online suppl. table 3 for the statistical analysis). In contrast to the results obtained for AMP gene expression, only three genes out of eleven showed the same type of transcriptional profile in both the systemic and bacteriome response when statistically analyzing the effects of time postinfection and of treatment. The weevil peptidoglycan recognition protein 1 (*wpgp1*), *pirk* and *relish*, which are homologous to the *D. melanogaster*-negative regulators *pgrp-lb* and *pirk*, and the NF- κ B transcription factor *relish*, respectively, were

significantly induced in both systemic and bacteriome responses following infection.

The expression of six of the eleven genes analyzed was either induced in the systemic response but not in the bacteriome, or not affected in the systemic response but repressed in the bacteriome. For example, the expression of genes encoding the putative receptors wPGRP3 and GNBPI (Gram-negative binding protein 1) significantly increased in the systemic response following infection. Interestingly, no significant induction was observed for these genes in the bacteriome. The expression of the *imd* gene was not affected in the systemic response but was repressed in the bacteriome at 2 and 6 h after both types of infection (i.e. with Gram-positive and Gram-negative bacteria), before returning to the level of the mock-infected control at 12 h. The *fk506bp*, which encodes an uncharacterized putative activator of the immune response through cytokine signaling [21] and an apoptosis inhibitor in vertebrates [22], was also downregulated after a Gram-negative infection in the bacteriome, whereas no significant changes were observed in the systemic response. The difference between the systemic and the bacteriome responses was even more striking in the case of *tollip*, a putative negative regulatory gene [23] that was induced by Gram-negative infection in the systemic response and repressed by both types of infection in the bacteriome, from 2 to 12 h after infection.

Finally, two genes were transcriptionally repressed in the systemic response but maintained, or even induced, in the bacteriome following infection. The expression of the regulatory gene *caudal* was not modified in the bacteriome after infection, but was slightly repressed by Gram-negative infection in the systemic response. The *toll* gene, encoding a membrane receptor of the Toll pathway, was repressed in the systemic response shortly after both types of bacterial infection. Its transcript level returned to the control level 12 h after treatment. Within the bacteriome, this gene expression was unchanged during the first hours following infection but was significantly induced 24 h after infection with Gram-negative bacteria. From these results, the bacteriome immune response appeared to be qualitatively and quantitatively different from the systemic immune response.

The Bacteriome Response Differs from the Gut Local Immune Response

Our data indicate major differences between the systemic and the bacteriome immune responses in *S. oryzae* larvae. Such quantitative and qualitative differences in AMP gene expression between the humoral and the so-called local immune response had already been described in *Drosophila* [24, 25]. The next step was to compare the bacteriome and the larval gut responses. The insect gut is known to generate a local immune reaction in response to oral bacterial infections [26, 27]. However, to our knowledge, whether the gut can also respond to systemic infection generated by a direct injection of bacteria into the hemocoel had not been previously investigated. We chose to analyze gene expression at 6 h following *D. dadantii* injections, when differences between the humoral and the bacteriome responses were the most striking. Expressions of all the genes monitored previously were measured in the gut of infected symbiotic individuals, except for those encoding the PGRP1, 2, 3 and the GNBPI proteins because their differential expression was only observed after at least 12 h postinfection in the systemic response study (fig. 5). *Sarcotoxin* and *coleoptericon B* were chosen as representative AMP genes. Our data showed that the expression of these AMP genes was significantly upregulated following bacterial infection in both tissues, and that their expression level was slightly, but significantly, higher in the gut than in the bacteriome (fig. 5). *Relish*, which encodes a homologue of the transcription factor that activates the Imd-dependent AMP expression in *Drosophila* [28], was also more highly expressed in the weevil gut when compared with the bacteriome. Its upregulation upon infection of the larva was significant in the bacteriome, supporting the results of the kinetics study. A similar upregulation tendency was observed in the gut, although it was not statistically significant (fig. 5). *pirk*, a homologue of the *Drosophila* *pirk* regulator, the expression of which is *relish*-dependent in the fruit fly [29, 30], showed higher expression in the gut when compared with the bacteriome (fig. 5). Finally, the *toll* gene, encoding for a Toll pathway receptor, showed no significant expression in the gut, but it was expressed and slightly induced in the bacteriome after injecting *D. dadantii*

Fig. 4. Expression kinetics of immune-related genes observed for humoral (H) and bacteriome (B) responses in larvae injected with *M. luteus* or *D. dadantii*. Whole aposymbiotic larvae were used to assess the systemic humoral immune response; symbiotic larvae were used to measure the bacteriome-specific response. Statistical results from the global two-way ANOVA analysis are displayed on

each graph with inf the infection type, t the time postinfection and int their interaction. $\bar{p} > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Detailed p values and full contrast analysis results are displayed in online supplementary tables 2 and 3. Error bars represent the standard error of four independent measurements.

(For figure see next page.)

Fig. 5. Expression of immune-related genes in the bacteriome (B) and gut (G) of symbiotic larvae 6 h after *D. dadantii* infection. Asterisks attached to gene names indicate a significant difference in global expression levels between B and G from a two-way ANOVA analysis. Asterisks on the bar plots indicate a significant difference

between infected and control conditions within one organ from a Welch two-sample t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Error bars represent the standard error of three independent measurements. PBS = PBS injected; Dd = *D. dadantii* injected.

(fig. 5). The remaining three genes analyzed, *imd*, *caudal* and *tollip*, showed no significant difference between the bacteriome and the gut. Overall, our data demonstrated that the bacteriome presents a specific immune signature that differs from both the systemic and the gut local immune responses.

The Bacteriome Response to Systemic Infections Does Not Affect Resident Endosymbiotic Bacteria

We have reported above that the bacteriome generates an immune response after systemic bacterial infection. This response is produced by an organ whose main function is to house and to tolerate endosymbiotic bacteria. Interactions with exogenous bacteria, and the subsequent immune response that they trigger, could be harmful for endosymbionts. To highlight any eventual noxious ef-

fects of the immune response on symbiosis, we quantified, by qPCR, the *S. pierantonius* endosymbionts over the course of a *D. dadantii* infection (fig. 6). Remarkably, the *S. pierantonius* load was not significantly different in infected larvae when compared with mock-infected larvae, suggesting that the bacteriome immune reaction does not harm the resident endosymbiotic bacteria.

Discussion

The interplay between innate immunity and endosymbiosis has been the focus of several studies over the last few years. In particular, questions concerning host tolerance to long-term symbiotic bacteria and the impacts of host-symbiont coevolution on the development

Fig. 6. *S. pierantonius* quantification in whole symbiotic larvae from 2 to 24 h after *D. dadantii* or *M. luteus* infection. The solid line represents the mock infection, the dashed line indicates infection with *D. dadantii* and the dotted line shows infection with *M. luteus*. No significant difference was found between infected and control larvae. Error bars represent the standard error of five independent measurements.

and adaptation of the host immune system remained unanswered [9, 11, 13, 31–33]. Remarkably, insects have selected a unique compartmentalization strategy that consists of secluding endosymbionts within the bacteriome. This seclusion limits endosymbiont exposure to host systemic responses and allows the expression of a specific cell program for symbiont maintenance and control inside the bacteriocytes. Transcriptomic studies have revealed that the bacteriome expresses specific immune genes involved in bacteriome immunomodulation and symbiont control [14, 31, 34]. In weevils, we have demonstrated recently that the bacteriome activates a limited number of immune effectors, including the AMP coleopteracin A [7]. The coleopteracin A peptide was shown to specifically target endosymbionts, inhibit their cell division and prevent endosymbiont externalization from this bacteria-bearing organ [9]. However, the bacteriome immune functions may not be limited to symbiont control. They may also include protection of both the bacteriocytes and their bacterial inhabitants against opportunistic exogenous pathogens. To address this question, we set up a model of systemic infection by *D. dadantii* in *S. oryzae* larvae. We have shown that *D. dadantii* was able to spread through the larval tissues within the initial 12-hour peri-

od following injection into the hemolymph. Nearly 50% of the infected individuals succumbed to *D. dadantii*, whilst surviving larvae succeeded in completely eliminating the bacteria 24 h after injection, not only demonstrating the efficiency of the weevil cellular and humoral immune responses, but also highlighting the variability between individual insects. At the initial stages of infection, *D. dadantii* was located in almost all the tissues, including the fat body, the gut and the bacteriome. At 12 h postinfection, a strong disparity was observed depending on how efficiently the larvae had coped with the infection. In dying larvae, *D. dadantii* had spread through all the tissues and the bacteria were massively associated with the gut epithelium and the bacteriome (fig. 2). In healthy-looking individuals, only a few bacteria were present in the fat body, suggesting that the systemic immune response was efficient enough to clear the infection. Remarkably, the remaining bacteria retreated between the fat body and the other insect tissues, including the gut and the bacteriome, but they did not succeed in penetrating them, presumably because of the local immune activity. At 24 h after infection, all the surviving larvae had completely eliminated *D. dadantii* and continued their development and metamorphosis into the adult stage.

Using the above model of infection, we showed that the weevil bacteriome generated an immune response following systemic infections with exogenous bacteria. This response includes the expression of various AMP genes. A comparison between the bacteriome and the systemic immune responses indicated that the same AMP genes were expressed in both cases in response to infections. Although levels of AMP gene expression attended to be higher after a Gram-negative infection than after a Gram-positive infection, the differences were not statistically significant. This suggests that either the larval immune system does not induce specific transcriptional responses to these two pathogen types, or that the differential responses rely on the transcription of genes that were not tested in this study.

Although qualitatively similar, the bacteriome and systemic immune response differ strongly in terms of the AMP gene induction levels, which were much higher in the systemic response than in the bacteriome. This is in line with previous analyses of local-type responses, such as the intestinal response to oral infection [26, 35].

Interestingly, when comparing AMP gene steady-state levels between the bacteriome and the gut 6 h after bacterial injection into the body cavity, we observed that AMP genes were activated in the gut following systemic infection. As far as we know, this is the first report of the acti-

vation of a gut immune response following an insect systemic infection. It indicates that the gut epithelium can mount an immune response not only to any bacteria present in the intestinal lumen, but also to bacteria present in the body cavity. AMP gene expression showed similar kinetics of induction and a similar order of magnitude between the gut and the bacteriome. However, AMP transcript levels were slightly, but significantly, higher in the gut, suggesting specific regulations of the immune response in both tissues. To investigate the bacteriome specificities further, we measured the transcript levels of genes homologous to the immune pathway components and regulators. Following infection, most of the genes studied were differentially expressed between the gut and the bacteriome responses, and five out of the eight genes analyzed were differentially expressed between the gut and the bacteriome after infection, including the *coleopteracin B*, *sarcotoxin*, *relish*, *pirk* and *toll* genes. In *Drosophila*, the local immune response is dependent on the activation of the IMD pathway, via the translocation of the NF- κ B transcription factor *relish* to the nucleus, resulting in the transcriptional activation of the AMP genes [26, 36]. *Pirk*, a negative regulator of the IMD pathway, is itself under the transcriptional control of *relish* [29, 30]. Since *relish*, AMP genes and *pirk* are all induced in the bacteriome following infection, we assume that at least one immune pathway is fully functional in the bacteriome, and that this pathway is closely related to the *Drosophila* IMD pathway. The fact that this IMD-like pathway is only slightly induced in the bacteriome, as compared with the gut, supports the hypothesis of a modulated immunity in this symbiotic organ. A functional analysis of weevil genes will now be required to test whether putative regulators assume a similar immunological function, as in the fruit fly immune pathways, and to determine their precise role in the bacteriome immune regulation.

Importantly, endosymbiont quantification showed no reduction in the size of the endosymbiotic community during the infection period compared with mock-infected controls. This indicates that the bacteriome immune response does not damage the resident endosymbiotic population, at least in terms of bacterial density. This effective protection of the endosymbionts during the course of an immune response could be partially due to the modulated, mild response generated by the bacteriome. An additional level of endosymbiont protection may rely on the existence of a predicted signal peptide in most weevil AMP gene sequences [7]. Secretion of bacteriome AMP to the basal border of the organ would allow endosymbionts to avoid contact with mature AMP. As

maintaining endosymbionts is essential for the weevil's physiological performance [37–39], it is likely that several functionally redundant mechanisms have been selected to ensure endosymbiont protection against the host immune responses.

To conclude, this work provides new insights regarding the bacteriome immune response to systemic infections. In parallel with the permanent immune response that targets endosymbionts [9], we have demonstrated that the bacteriome generates an inducible local-like immune response when the host is challenged with a systemic infection involving exogenous bacteria. This response may prevent pathogens from entering the bacteriome and competing with the endosymbionts. Qualitatively, the bacteriome response is relatively similar to the gut local response but it exhibits a lower activation level of AMP gene expression, which may prevent any harmful effects on the endosymbiotic population. These data suggest that the bacteriome has developed a specific, fine-tuned immune response with the dual function of protecting the resident endosymbiotic population from both exogenous infectious bacteria and from the host immune response itself. In this way, host-symbiont coevolution would seem to have developed a form of bacteriome immunity that preserves and protects the endosymbionts.

Acknowledgments

This work was supported by INRA, INSA-Lyon and the French ANR-10-BSV7-170101-03 (ImmunSymbArt) and ANR-13-BSV7-0016-01 (IMetSym). We would like to thank V.E. Shevchik and G. Condemine for supplying the A470 *D. dadantii* strain, and F. Subtil for his help and advice regarding the statistical analysis.

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