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# Efficient compartmentalization in insect bacteriomes protects symbiotic bacteria from host immune system

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## Abstract

**Background.** Many insects house symbiotic intracellular bacteria (endosymbionts) that provide them with essential nutrients, thus promoting usage of nutrient-poor habitats. Endosymbiont seclusion within host specialized cells, called bacteriocytes, often organized in a dedicated organ, the bacteriome, is crucial in protecting them from host immune defenses while avoiding chronic host immune activation. Previous evidence obtained in the cereal weevil *Sitophilus oryzae* has shown that bacteriome immunity is activated against invading pathogens, suggesting endosymbionts might be targeted and impacted by immune effectors during an immune challenge. To pinpoint any molecular determinants associated with such challenges, we conducted a dual transcriptomic analysis of *S. oryzae*'s bacteriome subjected to immunogenic peptidoglycan fragments.

**Results.** We show that upon immune challenge the bacteriome actively participates in the innate immune response via an induction of antimicrobial peptides (AMPs). Surprisingly, endosymbionts do not undergo any transcriptomic changes, indicating that this potential threat goes unnoticed. Immunohistochemistry showed that TCT-induced AMPs are located outside the bacteriome, excluding direct contact with the endosymbionts.

32 **Conclusions.** This work demonstrates that endosymbiont protection during an immune challenge is mainly  
33 achieved by efficient confinement within bacteriomes, which provides physical separation between host  
34 systemic response and endosymbionts.

35

## 36 Keywords

37 Symbiosis, immunity, bacteria, antimicrobial peptides, coleoptera, TCT

## 38 Background

39 Nutritional symbiosis between animals and microorganisms is a major driver of adaptation [1] as it  
40 participates in the colonization of nutrient-poor environments by complementing the metabolic needs of the  
41 host [2]. Notably, thanks to intracellular symbiotic bacteria (endosymbionts), insects can thrive on unbalanced  
42 carbohydrate-based diets, including blood, plant sap, or cereal grains [1,3–6]. However, the constant presence  
43 of microorganisms within an insect’s body represents a permanent challenge for the immune system [7]. The  
44 host immune system must conserve its ability to react against pathogens, while keeping beneficial symbionts  
45 alive and metabolically active [8]. The establishment of an equilibrium between excessive host colonization  
46 by the symbiont and chronic activation of the host immune system is essential in such symbiotic relationships,  
47 as the former would be detrimental for host survival, while the latter would result in symbiotic damage and  
48 host fitness reduction [9]. To better understand the co-evolution between the host immune system and the  
49 intracellular symbiotic bacteria, it is therefore important to pinpoint the molecular determinants of  
50 endosymbiont tolerance and pathogen control.

51 The association between the cereal weevil *Sitophilus oryzae* and its recently-acquired Gram-negative  
52 intracellular bacterium, *Sodalis pierantonius* (~28K years, [10,11]), is a remarkable example of homeostasis  
53 between insects and endosymbionts. *S. pierantonius* are contained within specialized gigantic cells, the  
54 bacteriocytes, which at the larval stages are located in a specialized organ – the bacteriome – at the foregut-  
55 midgut junction [3,12]. While wild *S. oryzae* animals are always associated with *S. pierantonius*, comparative  
56 studies between symbiotic and artificially-obtained aposymbiotic insects have shown that the presence of the  
57 endosymbiont accelerates insect development, allows strengthening of the insect cuticle [13], and enables  
58 flying [14].

59 Contrary to most long-lasting insect endosymbionts, the *S. pierantonius* genome contains genes encoding  
60 a functional type III secretion system (T3SS) [15], which was shown to be necessary during insect  
61 metamorphosis, where host stem cells are infected by the endosymbiont, followed by bacteriocyte  
62 differentiation and adult bacteriome formation [16]. The *S. pierantonius* genome also encodes genes necessary  
63 for Microbial-Associated Molecular Patterns (MAMPs) synthesis, including peptidoglycans (PGs), which are

64 able to activate the insect immune responses through their interaction with host pattern recognition receptors  
65 [7]. Injection of *S. pierantonius* into the insect hemolymph triggers the production of a plethora of  
66 antimicrobial peptides (AMPs) [17], suggesting its presence within the host body is an ongoing immune threat.  
67 Nevertheless, chronic immune system activation is avoided by the compartmentalization of the endosymbiont  
68 within bacteriocytes and the expression of an adapted local immune system [17–20]. The coleoptericin A  
69 (ColA) antimicrobial peptide (AMP) is an important molecular determinant for the maintenance of *S. oryzae*/*S.*  
70 *pierantonius* homeostasis. By interacting with the bacterial chaperonin GroEL, ColA inhibits bacterial cell  
71 septation and generates elongated bacteria with multiple genome copies [18]. Inhibition of *colA* with RNA  
72 interference leads to bacterial escape from the bacteriome, and colonization of host surrounding tissues [18].  
73 ColA expression in the bacteriome is dependent on *relish* and *imd*, two genes belonging to the immune  
74 deficiency (IMD) pathway [21]. Recently, the weevil's peptidoglycan recognition protein LB (PGRP-LB) was  
75 also shown to play a central role in host homeostasis. By cleaving the tracheal cytotoxin (TCT), a monomeric  
76 form of DAP-type peptidoglycan constantly produced by the endosymbionts within the bacteriome, PGRP-LB  
77 prevents the exit of TCT from the bacteriome to the insect's hemolymph, avoiding a chronic activation of host  
78 IMD dependent humoral immunity [19]. Taken together, these results suggest that bacterial  
79 compartmentalization in the bacteriome is a key strategy that allows the tolerance of symbiotic bacteria as it  
80 avoids the contact between the endosymbionts and the insect's immune system [22], therefore preventing  
81 chronic activation of the host immune IMD pathway against the beneficial microorganisms [23].

82 Current knowledge of gene expression levels in the larval bacteriome is limited to a couple of AMPs and a  
83 few other stress-related insect genes [19–21], and little is known about other insect or bacterial regulatory  
84 mechanisms involved in endosymbiont protection from bacteriocyte immune activation. We have previously  
85 shown that the bacteriome participates in the immune response against pathogenic bacteria and TCT challenge.  
86 Notably, up-regulation of several AMPs in weevils after injection of bacteria into the insect hemolymph is  
87 observed in the bacteriome [17,20], as well as in the rest of the body [17,20,24]. In addition, TCT injection is  
88 sufficient to mimic AMP induction in larval bacteriomes upon bacterial challenge [19]. It is important to note  
89 that AMP induction upon TCT challenge is IMD dependent, as is the control of endosymbionts within  
90 bacteriocytes, indicating the same pathway can fight exogenous bacterial infection while controlling  
91 intracellular beneficial bacteria [21]. Although the involvement of the bacteriome in the immune response  
92 would appear in disagreement with its primary function of hosting bacteria, such activation of the immune  
93 response against external infections does not seem to pose a threat to *S. pierantonius* integrity since bacterial  
94 infections do not induce a reduction in the number of symbionts [20]. This suggests that, despite activating the  
95 same immune pathway, differences must exist between fighting external infections and protecting the  
96 intracellular symbiont. We hypothesize that either the endosymbionts have evolved specific mechanisms to  
97 counteract the bacteriome immune response, or that host-controlled mechanisms, such as AMP secretion,  
98 ensure endosymbiont protection.



99 In this work, we conducted a global dual transcriptomic analysis of host bacteriomes and bacteria  
100 challenged systemically with TCT, in order to mimic an immune response in the absence of a real infectious  
101 threat. While confirming the involvement of the bacteriome in the immune response, notably via an AMP  
102 induction, immunohistochemical observations showed AMP accumulation only outside of the bacteriome, and  
103 a full preservation of the basal bacterial transcriptional program. Thus, efficient physical separation between  
104 symbionts and bacteria-harnessing molecules ensures full symbiont protection during an immune challenge.

## 105 Methods

### 106 Animal rearing, peptidoglycan challenge, and sample preparation

107 *S. oryzae* laboratory strain (Bouriz) were reared on wheat grains at 27.5°C and at 70% relative humidity. A  
108 strain of aposymbiotic insects was obtained as previously described [25]. The DAP-type peptidoglycan  
109 fragment TCT was purified from *Escherichia coli* as previously described [26]. Fourth instar larvae were  
110 extracted from wheat grains and challenged with a 0.2 mM TCT solution diluted in 1X phosphate buffered  
111 saline (PBS) injected into the hemolymph using a Nanoject III (Drummond). Sterile phosphate buffered saline  
112 PBS was also used as a negative control. Injected and non-injected larvae (naïve) were kept in white flour for  
113 6 hours at 27.5°C and at 70% relative humidity before dissection. Bacteriomes were dissected in  
114 diethylpyrocarbonate-treated Buffer A (25 mM KCl, 10 mM MgCl<sub>2</sub>, 250 mM sucrose, 35 mM Tris/HCl, pH =  
115 7.5). For each sample, bacteriomes were pooled (30 for Dual RNA-seq library preparation, and at least 25 for  
116 RT-qPCR), and stored at -80 °C prior to RNA extraction. Pools of five carcasses from symbiotic dissected  
117 weevils were used for RT-qPCR. Aposymbiotic samples consisted in pools of five fourth instar aposymbiotic  
118 larvae, which were torn in Buffer A, but not dissected as they do not harbor bacteriomes.

### 119 RNA extraction, library preparation and sequencing

120 Total RNA was extracted with TRIzol™ Reagent (Invitrogen, ref.: 15596026) following the manufacturer's  
121 instructions. Nucleic acids were then purified using the NucleoSpin RNA Clean up kit (Macherey Nagel, ref.:  
122 740948). Genomic DNA was removed from the samples with the DNA free DNA removal kit (Ambion, ref.:  
123 AM1906). Total RNA concentration and quality were checked using the Qubit Fluorometer (ThermoFisher  
124 Scientific) and TapeStation 2200 (Agilent Biotechnologies). Ribo-depletion and Dual RNA-seq strand-specific  
125 cDNA libraries were obtained starting from 100 ng of total RNA using the Ovation Universal RNA-seq System  
126 (NuGEN) following the manufacturer's instructions. Libraries were sequenced on a Nextseq 500 sequencer  
127 (Illumina), using the NextSeq 500/550 High Output Kit (Illumina).

### 128 Preprocessing, mapping of reads and differential expression analysis

129 Raw reads were processed using Cutadapt v1.18 [27] to remove adapters, filter out reads shorter than 50  
130 bp and reads that had a mean quality value lower or equal to 30. Clean reads were mapped against the *S. oryzae*  
131 genome (Genbank: PRJNA431034) with STAR v2.7.3a [28], and against the *S. pierantonius* genome  
132 (Genbank: CP006568.1) with Bowtie 2 v2.3.5 [29] with default parameters. Shared reads between the two  
133 genomes were filtered out with the aid of SAMtools v1.10 [30] and Picard v2.21.6 (available from  
134 <https://broadinstitute.github.io/picard/>). Gene counts were obtained for uniquely mapped reads with  
135 featureCounts v1.6.4 method from the Subread package [31]. Whenever uniquely mapped read counts were  
136 set to zero due to duplicated regions or multi-mapped reads, we further verified these regions within the multi-  
137 mapped read counts available with featureCounts. Insertion sequence (IS) families from the bacteria were also  
138 counted with the use of TETools (v1.0.0) with default parameters [32]. Gene counts and TETools counts were  
139 used as input for differential expression analyses using the DESeq2 v1.26.0 [33] package in R. After testing,  
140 the p-values were adjusted with the Benjamini-Hochberg correction [34] for multi-testing. Genes were  
141 considered differentially expressed when adjusted p-values (p-adj) were smaller than 0.05. Sequencing data  
142 from this study have been deposited at the National Center for Biotechnology Information Sequence Read  
143 Archive, <https://www.ncbi.nlm.nih.gov/sra> (accession no. PRJNA816415).

#### 144 Quantitative RT-PCR

145 Total RNA was extracted from fourth instar bacteriomes and carcasses, as well as from whole aposymbiotic  
146 fourth instar larvae using the RNAqueous - Micro kit (Ambion). DNA was removed with DNase treatment  
147 and RNA quality was checked with Nanodrop (ThermoFisher Scientific). Complementary DNA (cDNA) was  
148 produced with the iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions and  
149 starting with 500 ng total RNA. Differential gene expression was assessed by quantitative real-time PCR with  
150 a CFX Connect Real-Time PCR Detection System (Bio-Rad) using the LightCycler Fast Start DNA Master  
151 SYBR Green I kit (Roche Diagnostics), as previously described [19], except for *dpt4*, for which the annealing  
152 temperature was reduced to 54.5 °C. Data were normalized using the ratio of the target cDNA concentration  
153 to the geometric average of two housekeeping transcripts: *glyceraldehyde 3-phosphate dehydrogenase*  
154 (LOC115881082) and *malate oxidase* (LOC115886866). Primers were designed to amplify fragments of  
155 approximately 150 bp. A complete list of primers can be found in Additional Table 1.

#### 156 Immunohistochemistry

157 Larval samples challenged with TCT or PBS were prepared for histological observations as described in [19].  
158 Briefly, samples were fixed in paraformaldehyde (PFA) 4%. After one day, the fixative was replaced by several  
159 washings with PBS before embedding the tissue in 1.3% agar, then dehydrated through a gradient of ethanol  
160 (EtOH) washes and transferred to butanol-1, at 4°C, overnight. Samples were then placed in melted Paraplast  
161 and 3 µm-thick sections were cut with a HM 340 E microtome (ThermoFisher Scientific). Sections were placed  
162 on poly-lysine-coated slides, dried overnight at 37°C, and stored at 4°C.

163 For AMP localization, samples were dewaxed twice in methylcyclohexane for 10 min, rinsed in EtOH 100°,  
164 rehydrated through an EtOH gradient and then placed in PBS with 1% Bovine Serum Albumin (BSA) for 30  
165 min. ColA rabbit primary polyclonal anti-serum (Login et al., 2011) at 1:200 dilution, and a Coleoptericin B  
166 (ColB) primary polyclonal anti-serum (Proteogenix, Schiltigheim-France) at 1:300 dilution in 0.1% BSA were  
167 used. Preimmune rabbit serum (J0) was used as a negative control for ColA anti-serum, and BSA 0.1% for  
168 ColB (purified antibody). Antibody specificity was checked by western blot. After 1 h incubation at room  
169 temperature in the dark, sections were washed with PBS containing 0.2% Tween. Samples were then incubated  
170 with anti-rabbit IgG, labeled with Alexa Fluor 488. This secondary antibody was applied for 1 h at room  
171 temperature, diluted at 1:500 in 0.1% BSA in PBS. The excess of secondary antibody was washed with PBS-  
172 Tween, rinsed with PBS and washed several times with tap water. Sections were then dried and mounted using  
173 PermaFluor™ Aqueous Mounting Medium (ThermoFisher Scientific), together with 4,6-diamidino-2-  
174 phenylindole (DAPI, Sigma-Aldrich) for nuclear staining (3 µg/ml of medium). Images were acquired using  
175 an epifluorescence microscope (Olympus IX81), under specific emission filters: HQ535/50 for the green signal  
176 (antibody staining), D470/40 for the blue signal (DAPI) and HQ610/75 for the red signal (unspecific  
177 autofluorescence from tissue). Images were captured using an XM10 camera and the CellSens Software (Soft  
178 Imaging System). Images were treated using ImageJ (release 1.47v).

## 179 Results and discussion

### 180 Dual RNA sequencing successfully yielded both insect and bacterial transcripts

181 To investigate bacteriome response to an immune challenge, we extracted *S. oryzae*'s fourth instar larvae  
182 (L4) from grains and injected them with TCT, a fragment of the DAP-type peptidoglycan produced by Gram-  
183 negative bacteria, including *S. pierantonius* [19] and recovered bacteriomes six hours post injection as  
184 previously described [20]. TCT injection is able to trigger a potent response without the interference of an  
185 exogenous infectious bacteria [21]. Control larvae were injected with PBS, or extracted from grains but not  
186 injected (See Figure 1). To obtain the transcriptomic profile of both the symbiont and the host, Dual RNA-seq  
187 was performed in triplicates and yielded from 105 to 140 M reads per library (Additional Table 2). The reads  
188 were cleaned from adapter sequences and low-quality reads, and around 85% of the raw reads were kept for  
189 further analyses. We subsequently mapped the clean reads against both genomes, and obtained ~65-80%  
190 unambiguously mapping to the genome of *S. oryzae*, and ~5-8% to the genome of *S. pierantonius*. In each  
191 library from 23 to 33 M reads were uniquely mapped against insect genes (Additional Table 3), whereas ~3 M  
192 reads were uniquely mapped against bacterial genes (Additional Table 4). These results depict an improvement  
193 from our previous study, which yielded ~0.4 M reads mapped against bacterial genes in the same  
194 developmental stage and similar sequencing depth [16].

195

### 196 Systemic TCT challenge triggers AMP induction within the bacteriome

197 Sixteen *S. oryzae* genes were detected as differentially expressed (DE;  $p\text{-adj} < 0.05$ ) six hours after the TCT  
 198 challenge in the bacteriome, with respect to the bacteriome of non-injected (naïve) or PBS-injected larvae  
 199 (Table 1, Additional Table 5). Among these, one gene was strongly down-regulated, four were mildly down-  
 200 regulated, and eleven were up-regulated in response to TCT.

201 Table 1: *S. oryzae* genes differentially expressed in TCT-challenged bacteriomes ( $p\text{-adj} < 0.05$ ) identified by  
 202 Dual RNA-seq.

Gene Information				Average expression (TPM)*			Log2 Fold Change	
Gene ID	Type	Gene Abbreviation	Protein Name	Naïve	PBS	TCT	TCT vs Naïve	TCT vs PBS
<i>LOC115882681</i>	Unknown	N/A	Uncharacterized	2.93	1.06	0.03	-6.941	-5.574
<i>LOC115888453</i>	Transcription Factor	<i>adf-1</i>	Transcription factor Adf-1 family	54.55	49.23	34.33	-0.696	-0.601
<i>LOC115881033</i>	Translation Initiation	<i>elifebp-2</i>	Eukaryotic translation initiation factor 4E-binding protein 2	256.09	249.36	162.63	-0.691	-0.706
<i>LOC115891903</i>	Transcription Factor	<i>nrbp</i>	Nuclear receptor-binding protein	117.12	128.47	83.03	-0.518	-0.600
<i>LOC115883362</i>	Transcription Factor	<i>znf-91</i>	Zinc finger protein 91-like	48.09	46.24	36.16	-0.436	-0.434
<i>LOC115877563</i>	ABC Transporter	<i>mrp-4</i>	Multidrug resistance-associated protein 4-like	1.41	1.30	3.44	1.277	1.335
<i>LOC115885681</i>	Growth Factor	<i>brx</i>	Barietin toxin	1.24	1.13	4.30	1.793	1.881
<i>LOC115886735</i>	Bacterial Recognition	<i>gnbp-1</i>	Beta-1,3-glucan-binding protein-like	7.07	8.72	37.97	2.411	2.052
<i>LOC115874620</i>	AMP	<i>col-A</i>	Coleopterucin-A	251.44	480.30	2116.35	3.030	2.040
<i>LOC115883884</i>	AMP	<i>lux</i>	Luxuriosin	7.34	6.01	60.94	3.042	3.256
<i>LOC115884866</i>	AMP	<i>glyr-amp</i>	Glycine-Rich AMP	11.10	12.88	120.87	3.410	3.165
<i>LOC115888387</i>	AMP	<i>srx</i>	Sarcotoxin	27.81	28.61	407.20	3.826	3.734 <sup>§</sup>
<i>LOC115877462</i>	AMP	<i>dpt-2</i>	Diptericin-2	40.45	63.50	731.07	4.131	3.425
<i>LOC115877463</i>	AMP	<i>dpt-3</i>	Diptericin-3	9.45	13.71	261.58	4.759	4.164

<i>LOC115874703</i>	AMP	<i>col-B</i>	Coleopteracin-B	1.97	3.42	86.46	5.386	4.538
<i>LOC115877465</i>	AMP	<i>dpt-4</i>	Diptericin-4	1.31	2.52	98.43	6.196	5.225

§: This transcript was below the significance of detection in one of the conditions due to an outlier; results were verified with EdgeR and we validated this as a DE gene after the qPCRs.

\* Average expression is provided in transcripts per million (TPM).

203

204 RT-qPCR experiments confirmed the TCT-dependent induction of all 11 up-regulated genes (Figure 2,  
205 Additional Figure 1). Eight of these genes encode AMPs and all possess a predicted signal peptide: *colA*  
206 (Coleopteracin A), Coleopteracin B (*colB*), Sarcotoxin (*srx*), Luxoriosin (*lux*), a Gly-rich AMP (*gly-rich AMP*),  
207 and three Diptericins (*dpt-2, 3* and *4*, Figure 2) [35]. This AMP induction is in agreement with previous reports,  
208 where AMPs induced in larvae by immune challenge included *colA* [17,20,21,24], *colB*, *srx* [20,21,24], *dpt*,  
209 *cecropin* and *defensins* [20,24]. In addition to the eight AMPs, genes encoding one Gram-negative binding  
210 protein (*gnbp-2*), a barietin-like toxin (*brx*) and a multidrug resistant protein (*mrp-4*) were also up-regulated  
211 in the bacteriome (Figure 3). These three genes have not been identified in previous studies. *gnbp-2* is likely  
212 involved in insect defense responses against Gram-negative bacteria [36,37] and, like AMPs, contains a  
213 predicted secretory sequence at the peptide N-terminus (SignalP 6.0 likelihood value of 0.9998). It is  
214 noteworthy that another member of the *gnbp-2* family was also shown to be up-regulated in *S. oryzae*  
215 bacteriome in response to a bacterial challenge in a previous study [24]. The barietin-like toxin likely acts as  
216 a toxin directed against bacteria [38], similarly to AMPs, and also contains a predicted secretory sequence in  
217 the N-terminal region (SignalP 6.0 likelihood value of 1.0). Finally, *mrp-4 like* is likely a transporter involved  
218 in secretion of toxin and/or regulating homeostasis against pathogens [39]. In contrast, none of the down-  
219 regulated bacteriome genes detected in the Dual RNA-seq were confirmed by RT-qPCR (Additional Figure  
220 1). These results might be explained by their less pronounced down-regulation as seen by a milder Log2FC.  
221 Moreover, Dual RNA-seq was obtained from total ribodepleted RNAs, while RT-qPCR was performed on  
222 polyadenylated mRNAs, which could contribute to the differences observed in these analyses.

223 To test whether the identified up-regulated genes were part of a bacteriome-specific response, we analyzed  
224 the expression of the same genes in TCT- or PBS-challenged carcasses of symbiotic insects as well as in TCT-  
225 or PBS-challenged aposymbiotic L4 (*i.e.* insects artificially devoid of symbionts, with no bacteriome, see  
226 *Methods* Section). We found that all eight up-regulated AMPs (Figure 2) and the other three up-regulated genes  
227 (Figure 3) were also induced in TCT-challenged symbiotic carcasses, and TCT-challenged aposymbiotic  
228 whole larvae. The steady-state gene levels in PBS injection were comparable between the three conditions,  
229 with the exception of *lux*, *dpt-3* and *srx*. Finally, in agreement with previous studies, these data show that the  
230 bacteriome induction is generally milder than the systemic response [20], but confirms the involvement of the  
231 bacteriome in the host immune response. Previous studies have shown that *colA* is chronically expressed in  
232 the larval bacteriomes, here seen at ~250 transcripts per million (TPM) in control conditions, and it successfully  
233 prevents endosymbiont escape and morphology (Login et al., 2011, Maire et al., 2019). The TCT-induced AMP  
234 upregulation in the bacteriomes might therefore constitute a threat for endosymbiont fitness. Overall, these

235 results strongly suggest that the presence of *S. pierantonius* does not affect the systemic induction of AMPs,  
236 which is comparable between symbiotic and aposymbiotic insects.

237 It is important to note that the present study failed to detect a couple of host genes previously identified as  
238 up-regulated upon bacterial infection in *S. oryzae*, including the regulatory gene *pirk*, and the Toll pathway-  
239 related genes (*pgrp*, *toll*), among others [20]. These discrepancies might indicate the inability of the TCT  
240 molecule to trigger a complete immune response, as opposed to a whole bacterium. TCT is a monomeric form  
241 of DAP-type PG and induces only the IMD and not the Toll pathway [19]. Nevertheless, the AMP induction  
242 observed here is consistent with previous studies [20,24] and would be expected to constitute a severe threat  
243 for the endosymbionts in the absence of protective mechanisms.

244

## 245 Symbiotic bacteria are insensitive to the activation of the bacteriome immune system

246 In order to identify potential signatures of bacterial stress and gene modulations to counteract the insect  
247 immune response and AMP induction, the symbiont transcriptomic profile obtained by Dual RNA-seq from  
248 TCT-challenged bacteriome samples was compared with controls, *i.e.* PBS-injected or naïve. Remarkably, the  
249 differential analysis revealed that bacterial transcription is unresponsive to the TCT challenge (Additional  
250 Tables 6). Furthermore, and similarly to coding regions, we did not detect changes in expression in repetitive  
251 regions (IS) (Additional Table 7). Moreover, a previous study using Dual RNA-seq in *S. oryzae* showed around  
252 400 differentially expressed bacterial genes throughout the metamorphosis of the insect, confirming the ability  
253 of the endosymbiont to modulate gene expression in response to host developmental stimuli [16]. The contrast  
254 between large changes of gene expression during metamorphosis, with a complete lack of differentially  
255 expressed genes upon TCT challenge, strongly suggests that the bacteria do not sense the AMP induction or  
256 any other stress induced by such challenge [42].

257 Rather, analysis of the complete bacterial transcriptome from both controls and TCT-challenged larvae  
258 display similar gene expression. Highly expressed bacterial protein coding genes detected within the  
259 bacteriome (Additional Table 8) are mainly involved in transcriptional regulation, translation, stress response,  
260 and virulence (see Additional Text 1 for more information). Several transcriptional, translational and  
261 stabilization factors of the general stress response sigma factor RpoS (reviewed in [40]) were similarly  
262 expressed at varied levels in all conditions (Additional Table 9). The expression of *rpoS* was lower than the  
263 vegetative sigma factor *rpoD*, which is a typical profile of the exponential growth phase in *Escherichia coli*  
264 [41]. This basal level of *rpoS* is also needed for triggering a fast stress response in diverse bacteria [40], and  
265 shows the ability of *S. pierantonius* from larval bacteriomes to quickly enter a "virulent mode" in the  
266 subsequent pupal stage that allows them to exit bacteriocytes and re-infect stem cells [16].

267 Together with previous findings that the symbiont population remains unchanged even after an immune  
268 challenge with pathogenic bacteria [20], this suggests that other regulatory mechanisms are in place to maintain  
269 the physical integrity of the symbiotic bacterial population during host AMP induction.

## 270 Mature AMPs are physically separated from endosymbionts



271 One of the hallmarks of AMPs is the presence of a N-terminal secretory sequence that addresses them to  
272 the outside of the cell, including the hemolymph, to counteract systemic infections [52]. Thus, even though  
273 cells in the bacteriome can produce AMPs, their final localization outside of bacteriocytes would ensure  
274 protection of the endosymbionts from AMP harm. However, in physiological conditions, ColA is produced by  
275 and retained inside the bacteriocytes, together with the endosymbionts, where it keeps them from escaping  
276 [18]. Since our knowledge of AMP localization is still limited because of the lack of specific antibodies, it  
277 cannot be excluded that other AMPs might also accumulate intracellularly, especially if highly expressed, and  
278 constitute a threat for the endosymbionts. We therefore assessed the localization of TCT-induced AMPs with  
279 respect to the symbionts. We performed immunohistochemistry with polyclonal antibodies able to recognize  
280 *colB*, an AMP previously shown to be induced by TCT and bacterial challenges [19] and the bacteriome-  
281 specific AMP ColA [18]. The choice of *colB*, in particular, was dictated by the fact that, despite this peptide  
282 being very similar to *colA* (46.72% of amino acid sequence identity), their function is remarkably different, as  
283 *colA* is expressed constitutively in the bacteriome where it interacts with GroEL and contributes to the insect-  
284 bacteria homeostasis. Samples were taken at six hours after the immune challenge with TCT or PBS (as for  
285 the transcriptomic analysis), so that we could confirm that AMPs were induced at the protein level, despite the  
286 lack of endosymbiont response. In the PBS-injected controls (Figure 4A-D), ColA was detected within the  
287 bacteriome (Figure 4A) - as expected because of its role in preventing symbiont escape (Login et al., 2011) -  
288 but not in the other tissues (Figure 4B). These results confirm the presence of ColA within the bacteriome,  
289 even at basal expression levels. In contrast, ColB was not detected inside the bacteriome (Figure 4C), nor in  
290 other surrounding cells, including gut tissues (Figure 4D). In response to TCT (Figure 4E-H), ColA was still  
291 clearly detectable within the bacteriome (Figure 4E), as expected, but also in several epithelial gut cells as well  
292 as in the acellular extended region that likely corresponds to the hemolymph (Figure 4F). This confirms the  
293 dual role of ColA in both symbiosis control [18] and in response to an exogenous immune challenge. On the  
294 contrary, ColB was still absent from the bacteriome tissue following TCT challenge (Figure 4G), but, similarly  
295 to ColA, was detected in the hemolymph of TCT-challenged larvae (Figure 4H).

296 The results show that, in agreement with the lack of endosymbiont transcriptomic response, the excess of  
297 ColA, ColB (and potentially all other AMPs (whether induced in the bacteriome or the fat body), remain  
298 physically separated from the endosymbionts. Thus endosymbiont integrity is protected even while AMPs are  
299 participating in the systemic immune response.

## 300 Conclusions

301 There are currently three main known strategies allowing symbiotic microorganisms to coexist with  
302 efficient and responsive insect immunity: *i*) evolution of the ability to differentiate between pathogenic and  
303 symbiotic MAMPs by the host, *ii*) bacterial molecular modifications leading to immune tolerance, notably  
304 promoting biofilm formation [53], and *iii*) compartmentalization of the symbionts in specialized symbiotic  
305 organs, often called bacteriomes [54]. The compartmentalization strategy sequesters the symbionts in



306 specialized cells, creating a favorable environment for their metabolic activity, and keeping them under control  
307 while avoiding overproliferation and virulence. The bacteriomes are therefore found in many insect species,  
308 including aphids [55], planthoppers [56], cicadas [57], and beetles [58]. Although very common, little is known  
309 about the evolution and immune modulation inside the bacteriomes, as well as on their formation and  
310 maintenance.

311 In the *S. oryzae/S. pierantonius* symbiosis, bacterial MAMPs are able to trigger a potent immune response,  
312 thus excluding a selective tolerance of the weevil immune system towards *S. pierantonius* MAMPs [17,19–  
313 21]. The absence of bacterial transcriptomic response to the systemic TCT immune challenge excludes active  
314 mechanisms of immune suppression from the endosymbiont. Rather, compartmentalization of *S. pierantonius*  
315 within bacteriomes guarantees physical separation between the endosymbionts and AMPs that might be  
316 produced by the bacteriome itself or elsewhere (*e.g.* fat bodies). This mechanism is crucial to protect both the  
317 host from the symbionts, and the bacteria from the insect immune system [18,21]. As demonstrated by the  
318 immunofluorescence labeling, there is no colocalization of endosymbiont-containing cells and AMPs, with the  
319 notable exception of ColA due to its homeostatic function, thus showing that not only the bacteriome acts as a  
320 physical barrier against the external AMPs, but is also capable to efficiently drain away the toxic molecules  
321 produced both inside or outside the bacteriome (Figure 5). Altogether these data refine the understanding on  
322 how an organ such as the bacteriome can ensure specific symbiotic function, *i.e.* maintain and control  
323 endosymbionts in a specific location, while potentially participating in the immune response to exogenous  
324 bacteria.

## 325 List of abbreviations

326 AMP (antimicrobial peptide), BSA (bovine serum albumin), cDNA (complementary DNA), DAPI (4,6-  
327 diamidino-2-phenylindole), EtOH (ethanol), IMD (immune deficiency), IS (insertion sequence), L4 (fourth  
328 instar larvae), MAMPs (microbial-associated molecular patterns), p-adj (adjusted p-values), PBS (phosphate  
329 buffered saline), PFA (paraformaldehyde), PG (proteoglycan), T3SS (type III secretion system), TA (toxin-  
330 antitoxin), TCT (tracheal cytotoxin), TPM (transcripts per million).

## 331 Declarations

332 Ethical approval and consent to participate

333 Not applicable

334 Consent for publication

335 Not applicable

336 Availability of data and materials

337 Sequencing data from this study have been deposited at the National Center for Biotechnology Information  
338 Sequence Read Archive, <https://www.ncbi.nlm.nih.gov/sra> (accession no. PRJNA816415).

### 339 Competing interests

340 The authors declare that they have no competing interests" in this section.

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### 345 Author's contributions

346 AH, CV and RR conceived the original project. EDA was responsible for all molecular biology methods,  
347 with the help of AV. AV in collaboration with SH and BG constructed the Dual RNA-seq libraries and  
348 produced the sequencing reads. MGF was responsible for the bioinformatic analyses of the Dual RNA-seq  
349 with the help of NP. EDA, MGF and RR analyzed the data. EDA with the help of SB performed the  
350 immunofluorescence experiments. EDA, MGF, RR wrote the manuscript with the help of CV, AH, CVM and  
351 AZR. All authors read and approved the final manuscript.

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360 @niparisot (Nicolas Parisot) and @rita\_rebollo (Rita Rebollo).

361

## 362 Figure Legends

363 Figure 1. Schematic diagram of the experimental design. Top left panel: image of a *S. oryzae* 4th instar larva  
364 along with a schematic section. Top left panels: symbiotic and aposymbiotic *S. oryzae* fourth instar larvae were

365 extracted from grains and dorsally injected with 0.2 mM PBS, 0.2 mM TCT at the level of the haemolymph.  
366 Other larvae were extracted from grains but not injected (Naïve). Bacteriomes and carcasses were sampled  
367 from PBS/TCT-injected or naïve symbiotic larvae alongside whole aposymbiotic larvae. Bottom panel: dual  
368 RNA-seq was performed to detect insect and bacterial expression profiles was performed on bacteriomes and  
369 carcasses of symbiotic weevils (PBS, TCT and Naïve samples). RT-qPCR experiments were performed on  
370 TCT and PBS-treated bacteriomes/carcasses from symbiotic weevils as well as whole larvae from  
371 aposymbiotic weevils, to detect bacteriome-specific and/or symbiont-dependent transcriptomic changes.

372  
373 Figure 2. Differential expression of TCT-induced AMPs in bacteriomes. The quantification was performed by  
374 RT-qPCR on *S. oryzae* bacteriomes and carcasses of symbiotic weevils, as well as on whole aposymbiotic  
375 larvae. Green dots: PBS-injected larvae (control); red squares: TCT-injected larvae. Asterisks denote statistical  
376 significance (ANOVA with Kruskal-Wallis test, \* =  $p \leq 0.05$ ). Error bars represent SE. Overall, the AMP  
377 induction in response to TCT is observed in both bacteriomes and carcasses of symbiotic weevils, as well as  
378 in aposymbiotic weevils.

379  
380 Figure 3. Differential expression of TCT-induced genes in bacteriomes other than AMPs. The quantification  
381 was performed by RT-qPCR on *S. oryzae* bacteriomes and carcasses of symbiotic weevils, as well as on whole  
382 aposymbiotic larvae. Green dots: PBS-injected larvae (control); red squares: TCT-injected larvae. Asterisks  
383 denote statistical significance (ANOVA with Kruskal-Wallis test, \* =  $p \leq 0.05$ ). Error bars represent SE.  
384 Overall, upregulation in response to TCT is observed in both bacteriomes and carcasses of symbiotic weevils,  
385 as well as in aposymbiotic weevils.

386  
387 Figure 4. AMP localization in *S. oryzae* larvae, before and after TCT immune challenge. Upper panel: ColA  
388 (first row) and ColB (second row) localization in PBS-injected larvae. Lower panel: ColA (first row) and ColB  
389 (second row) localization in TCT-injected larvae. Ba: bacteriome; GL: gut lumen. Asterisks indicate  
390 accumulation of AMPs in the hemolymph. Scale bar: 50  $\mu\text{m}$ .

391  
392 Figure 5. Proposed mechanisms of TCT challenge response within bacteriomes of *S. oryzae*. TCT injected in  
393 the hemolymph reaches bacteriomes and is recognized by PGRP-LC from bacteriocytes. Through a signaling  
394 cascade potentially dependent on IMD/RELISH proteins, bacteriocytes activate an AMP induction which for  
395 the most part are thought to be secreted (ColB, Srx, Lux, Gly-Rich AMP, Dpt-2, -3, and 4) to aid in the global  
396 immunity response, but no effectors are perceived by the bacteria within bacteriomes. AMPs are also produced  
397 by the fat body and secreted to the hemolymph. ColA in turn is kept within bacteriocytes to prevent *S.*  
398 *pierantonius* from exiting the host cells during this immune challenge.

399  
400

## 401 Additional Files

402 Additional Figure 1. Differential expression of TCT-repressed genes in bacteriomes, according to Dual RNA-  
403 seq. The quantification was performed by qRT-PCR on *S. oryzae* bacteriomes and carcasses of symbiotic  
404 weevils, as well as on whole aposymbiotic larvae. Green dots: PBS-injected larvae (control); red squares: TCT-  
405 injected larvae.

406 Additional Text 1: Description of genes highly expressed from *S. pierantonius*.  
407 Additional Table 1: Primer sequences

408 Additional Table 2: Dual RNA-seq trimming and mapping statistics.

409 Additional Table 3: Count data of *S. oryzae* genes

410 Additional Table 4: Differential expression analysis of *S. oryzae* genes.

411 Additional Table 5: Level of expression of *S. pierantonius* genes.

412 Additional Table 6: Differential expression of *S. pierantonius* genes.

413 Additional Table 7: Count data and differential expression analysis of *S. pierantonius* ISs.

414 Additional Table 8: Highly expressed bacterial genes in all conditions (TPM > 1000) belonging to key  
415 biological functions in *S. pierantonius*.

416 Additional Table 9: Expression levels of genes related to the general stress response in *S. pierantonius*.

417

418

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## Model system

*S. oryzae*

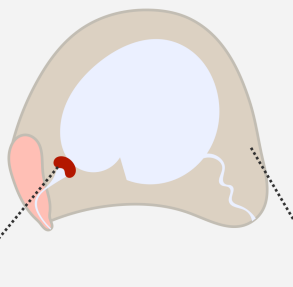
4th instar larvae



ant.

post.

Schematic section

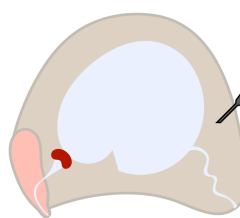
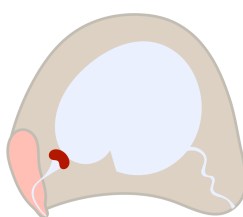


Bacteriome

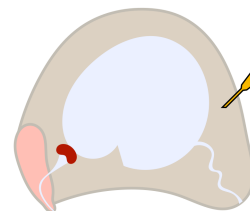
Carcass

## Experimental conditions

Naïve



PBS



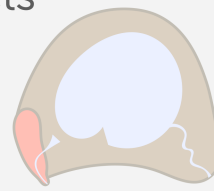
TCT

## Dissection

Symbiotic insects



Bacteriome



Carcass

Aposymbiotic insects



Whole insect

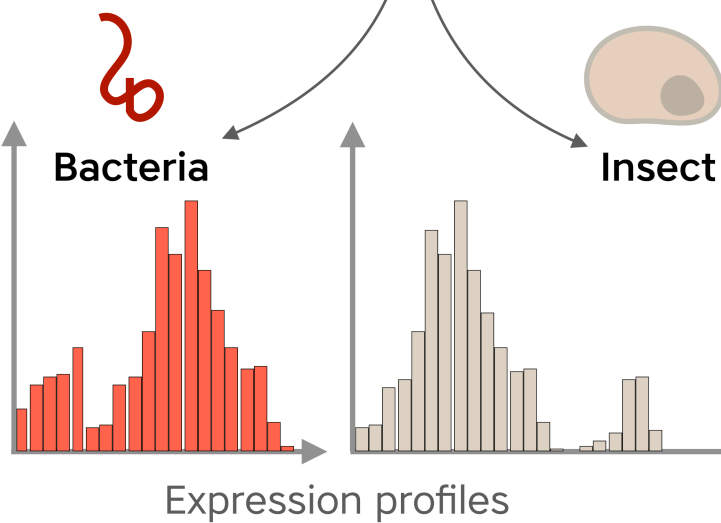
Dual RNA-sequencing

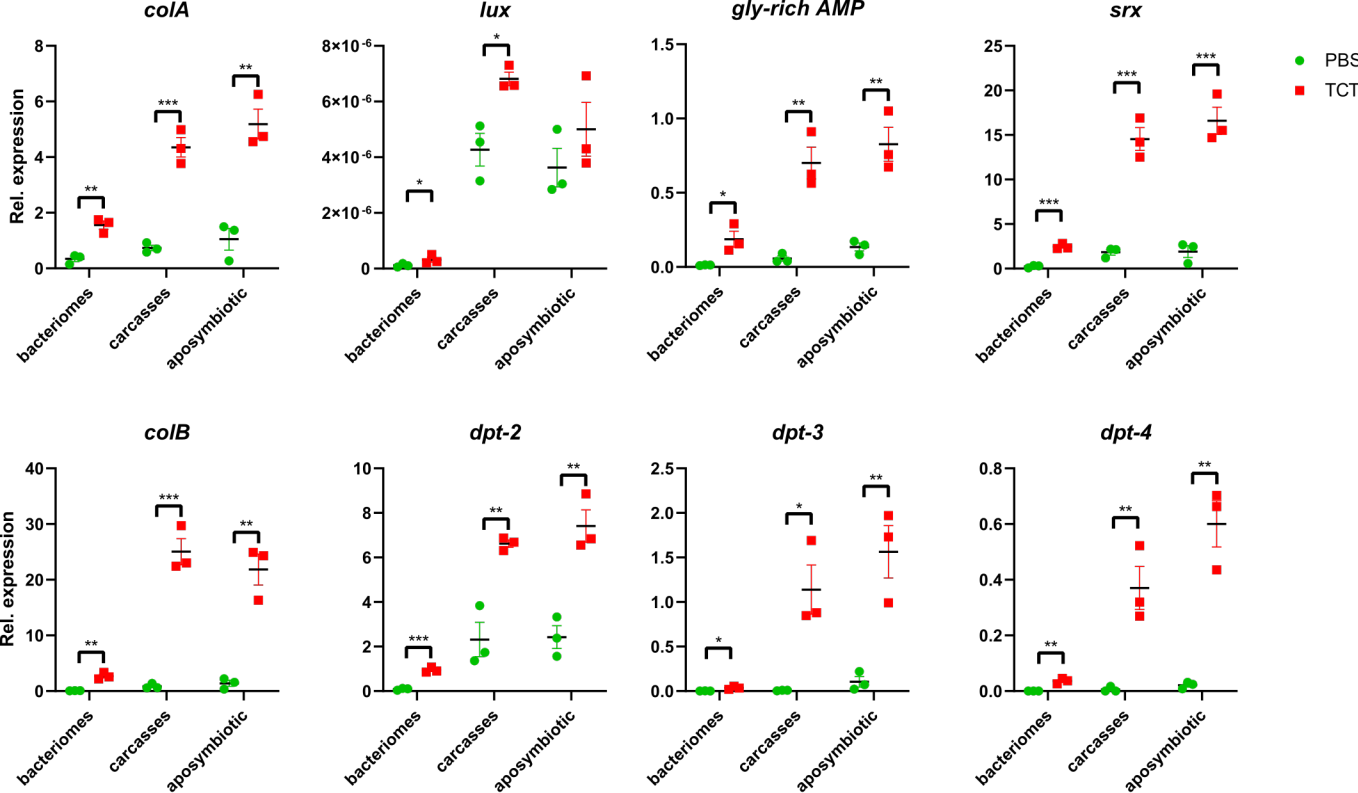
Selected genes

RT-qPCR

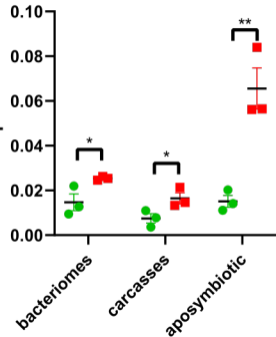


Experimental design

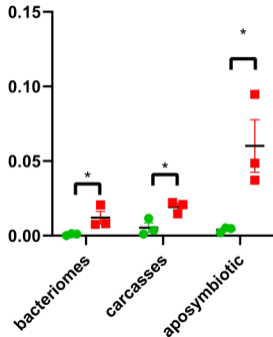




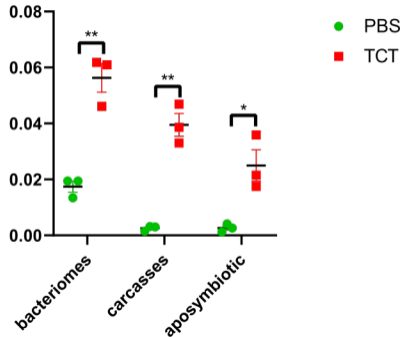
*mrp-4* like



*brx*

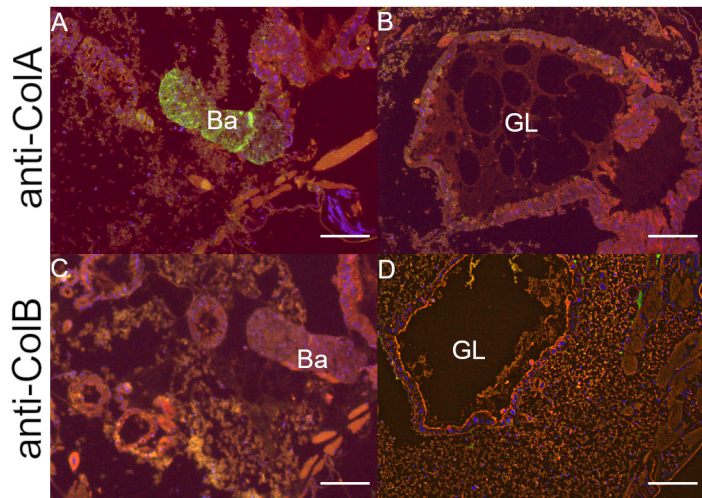


*gnbp-2*



● PBS  
■ TCT

PBS



TCT

