Dissertation

Identification of the *Escherichia coli* gene *nlpI* whose expression is enhanced by a host immune receptor

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SUMMARY

Interaction between the host and pathogen determines the fate of both organisms during the infectious state. The host is equipped with a batterry of immune reactions, while the pathogen displays a variety of mechanisms to compromise host immunity. Although bacteria alter their pattern of gene expression in host organisms, studies to elucidate the mechanism behind this are only in their infancy. I here examined the possibility that host immune proteins directly participate in the change of gene expression in bacteria. *Escherichia coli* was treated with a mixture of the extracellular region of peptidoglycan recognition protein (PGRP)-LC and the antimicrobial peptide attacin of *Drosophila melanogaster*, and subjected to a DNA microarray analysis for mRNA repertoire. I identified 133 annotated *E. coli* genes whose mRNA increased after the treatment. One such gene, lipoprotein-encoding *nlpI*, showed a transient increase of mRNA in adult flies depending on PGRP-LC. NlpI-lacking *E. coli* had a lowered growth rate and/or viability in flies than the parental strain. These results suggest that a host immune receptor triggers a change of gene expression in bacteria simultancously with their recognition and induction of immune responses.

INTRODUCTION

Functional interaction between host organisms and invading microbes determines the fate of infection, that is, the disappearance of the invaders or the development of diseases. Upon infection, the host activates immunity to eliminate pathogenic microbes, while the invader exerts a variety of strategies to resist this (1-3). In most cases, these responses on both sides are accompanied by a change of gene expression pattern, mainly at the transcription step. Host organisms recognize the existence of microbes through the surveillance mechanism performed by immune factors, in particular those constituting innate immunity (4-6). Various cellular as well as humoral materials are involved in sensing microbes and subsequently inducing innate immune reactions, exemplified by the production of antimicrobial substances. On the other hand, microbes activate the expression of a set of genes whose products mitigate the host immune response, although the underlying mechanism is largely unknown (1).

Bacteria adapt themselves to new environments, many of which are hostile, by altering the pattern of gene expression. There are well-known mechanisms for transcriptional control of bacterial genes in response to environmental changes; namely, the two-component regulatory system (7,8), quorum sensing (9,10), and the clustered regularly

interspaced short palindromic repeat (CRISPR)-Cas system (11-13). As a result of these mechanisms, bacteria alter the usage of genes to control their behavior. Importantly, the involvement of the two-component regulatory system (14) and the CRISPR-Cas system (15) in the control of bacterial virulence has been reported.

I anticipated the presence of another mechanism by which bacterial gene expression is altered during bacterium-host interaction. The invasion of bacteria is recognized by the host through the molecular interaction between host immune receptors and bacterial substances constituting the cell wall, which evokes the induction of an array of immune reactions including the production of antimicrobial substances and the phagocytic killing of bacteria. This means that cell wall components serve as ligands to activate receptors that exist either at the surface of immune cells or as soluble proteins. I hypothesized that the opposite could be true: receptors of host immune cells function as ligands to activate cell wall components that serve as receptors for the induction of bacterial genes (Fig. 1).

In mammals, various structures of bacterial cell wall components, often called the pathogen-associated molecular pattern, are bound by a narrower set of immune proteins called pattern-recognition receptors or Toll-like receptors (16). In insects such as *Drosophila melanogaster*, a single component of the cell wall, namely, peptidoglycan, plays a major role as a ligand to stimulate host receptors called peptidoglycan recognition protein

(PGRP) for activation of the nuclear factor (NF)-κB-mediated transcription of immunity-related genes including those coding for antimicrobial peptides (17). There are two types of bacterial peptidoglycan that differ in the amino acid residue at the third position in the stem peptide: one with lysine present in most Gram-positive bacteria and the other with meso-diaminopimelic acid (DAP) present in Gram-negative bacteria and Gram-positive bacili (18). In *Drosophila*, lysine-type peptidoglycan is recognized by PGRP-SA and PGRP-SD and elicits activation of the Toll pathway, while DAP-type peptidoglycan is bound by PGRP-LC and PGRP-LE leading to induction of another pathway, namely, the IMD pathway (18-20). The Toll and IMD pathways are responsible for the transcription of most immune-responsive genes activated upon infection with bacteria and fungi (21) (Fig. 2).

To validate the above described hypothesis, I took advantage of *Drosophila*, a model animal simpler than mammals with regard to the study on innate immunity, and examined whether the pattern of gene expression changes in bacteria when they are exposed to PGRP. Data from a series of biochemical and genetic experiments indicated that this is the case.

MATERIALS AND METHODS

Fly stocks and bacterial strains

The lines of *Drosophila* w^{1118} used as a control, *PGRP-LC*^{7454} lacking PGRP-LC (22) (a gift from J. Royet), *PGRP-LE*^{112} lacking PGRP-LE (23) (a gift from S. Kurata), and *PGRP-LE*^{112}*;PGRP-LC*^{7454} lacking both PGRP-LC and PGRP-LE (23) (a gift from S. Kurata) were used. The *E. coli* K-12 strain BW25113 and its derivative JW3132 (*nlp*-deficient mutant) were obtained from the Keio Collection, a library of *E. coli* with deletions in the open-reading frame of individual genes (24) (National BioResource Project: National Institute of Genetics, Mishima, Japan). The fly lines and bacterial strains used in this study are listed in Table 1.

Preparation of *Drosoplila* immune proteins

The extracellular region of 3 subtypes of PGRP-LC, namely, a (amino acid positions 313-520), x (amino acid positions 313-500), and y (amino acid positions 313-511), and the antimicrobial peptide attacin were all prepared recombinantly as proteins fused to glutathione-*S*-transferase (GST) at the N-terminus (Fig. 3) (GST-attacin, GST-PGRP-LC). GST-PGRP-LCx and GST-PGRP-LCy were expressed in insect Sf9 cells using a

baculovirus-based vector system (Life Technologies Japan, Tokyo, Japan), and affinity-purified by glutathione-Sepharose chromatography (GE Healthcare Japan, Tokyo, Japan), essentially as described previously (25). GST-PGRP-LCa, GST-attacin, and GST alone were expressed in *E. coli* using the vector pGEX-KG and purified as described above. In the preparation of GST-PGRP-LCa and GST-attacin, bacterial lysates were treated with guanidinium chloride to solubilize possible inclusion bodies followed by dialysis against 50 mM Tris-HCl, pH 7.5 to allow proteins to refold prior to affinity chromatography. The purity of the recombinant proteins was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue R-250 or Western blotting with anti-GST monoclonal antibody (Merck Millipore, Tokyo, Japan) and horse radish peroxidase-conjugated anti-mouse immunoglobulin (Ig) G antibody (GE Healthcare Japan).

Assay for binding of GST-fused proteins to peptidoglycan

A solid-phase binding assay was conducted essentially as described previously (26). In brief, dishes of a 96-well culture container (MS-8496F; SUMITOMO BAKELITE, Tokyo, Japan) were coated with peptidoglycan (3 μg per well) of *E. coli* K-12 strain (PGN-EK; InvivoGen, San Diego, CA, USA) that had been partially solubilized by sonication. A

mixture of GST-PGRP-LCx, GST-PGRP-LCa, and GST-PGRP-LCy, or GST alone as a negative control were added to the wells in triplicate, incubated for 3 h at room temperature, washed, and successively reacted with anti-GST monoclonal antibody (Merck Millipore) and horse radish peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare Japan). The samples were finally subjected to a colorimetric reaction using *0*-phenylenediamine as a substrate, and the amount of the reaction products was determined by measuring A_{490}.

DNA microarray analysis

The *E. coli* strain BW25113 (2×10^9) that had grown with Luria-Bertani medium to the stationary phase of cell growth was suspended with insect saline (27) (0.13 M NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) and incubated with a mixture of GST-attacin (0.125 μM), GST-PGRP-LCa (0.5 μM), GST-PGRP-LCx (1 μM), and GST-PGRP-LCy (0.5 μM) for 10 min at room temperature. As a negative control, incubation of *E. coli* was carried out in the presence of GST alone (3 μM). The bacteria were subjected to total RNA extraction using RNeasy Micro Kit (Qiagen, Tokyo, Japan), and resulting RNA was used to synthesize cDNA with a 6-base random primer (Life Technologies Japan) and reverse transcriptase (Superscript Ⅱ; Life Technologies Japan). The cDNA was purified using MinElute PCR Purification Kit (Qiagen), fragmented into 50～200 base pairs with DNase Ⅰ (Takara-Bio,

Kyoto, Japan) (0.06 units/μg DNA), labeled with biotin using terminal deoxynucleotidyl transferase (Promega KK, Tokyo, Japan) and GeneChip Labeling Reagent (Affymetrix, Santa Clara, CA, USA), and used as a target for hybridization with a probe on a GeneChip (*E. coli* Genome 2.0 Array; Affymetrix). Hybridization was carried out at 45 ℃ for 16 h, and the GeneChip was washed and incubated with streptavidin-phycoerythrin. Signal acquisition was carried out using Affymetrix GeneChip System with Affymetrix GeneChip Command Console software. All the microarray data are Minimum Information About a Microarray Experiment (MIAME)-compliant and have been deposited in an MIAME-compliant database, the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, Gene Expression Omnibus Series accession number GSE61604), as detailed on the website of the Microaray Gene Expression Data Society (http://www.mged.org/Workgroups/MIAME/miame.html).

The original CEL files were quantified with the Distribution Free Weighted method (28) (DFW) using statistical language R (http://www.r-project) (29) and Bioconductor (http://www.bioconductor.org/) (30). Hierarchical clustering was performed using the pvclust() function (31) in R. To identify differentially expressed genes (DEGs), the rank products method (32) was applied to the data quantified using DFW with the number of permutations set at 500. Probe sets with a false discovery rate (FDR) <0.05 were regarded

as having different expression levels between the two groups (i.e., they were differently expressed). The annotation file for the *E. coli* Genome 2.0 Array was obtained from the Affymetrix website (October 29, 2012, E_coli_2.na33.annot.csv). A gene-annotation enrichment analysis of DEGs was performed using the Database for Annotation, Visualization, and Integrated Discovery (http://david.abcc.ncifcrf.gov/) (33) and Quick GO (http://www.ebi.ac.uk/QuickGO/) (34). Expression Analysis Systematic Explorer Scores, which are modified Fisher`s exact test *p* values (35), were used to extract statistically overrepresented Gene Ontology (GO) terms form the DEGs. GO terms with *p* values of <0.05 were regarded as significantly enriched.

Semi-quantitative reverse transcription-mediated polymerase chain reaction

Toal RNA extracted from *E. coli* or *E. coli*-infected adult flies using RNeasy Micro Kit was used as a template in reverse transcription with a 6-base random primer, and the resulting cDNA was used as a template for polymerase chain reaction (PCR) with primers corresponding to individual genes to be analyzed. The nucleotide sequences of DNA oligomers used as primers in PCR are shown in Table 2. The amplified DNA was separated by 6% (w/v) polyacrylamide gel electrophoresis and visualized by staining with ethidium bromide. Messenger RNA of RpoA, the α-subunit of *E. coli* RNA polymerase, was

analyzed as an unchanged control and used to normalize the data for other mRNA (14) : the amount of cDNA from different RNA samples was adjusted so that the same intensity of signal derived from RpoA mRNA was obtained prior to the analysis of other mRNA.

Infection of adult flies with bacteria and colony-foming assay

E. coli was culturred at 37 ℃ with Luria-Bertani medium, harvested at the stationary phase of cell growth, washed with insect saline, and re-suspended with insect saline. Male adult flies were infected with *E. coli* in the hemocoel according to the established method (36) with modification (37). In brief, flies (10 flies for mRNA analysis and 5-10 flies for colony-forming assay) were injected at the abdomen with *E. coli* suspended with 50 nl of insect saline (2.5×10^6 for mRNA analysis and 3×10^6 for colony-forming assay) with the aid of a nitrogen gas-operated microinjector (Narishige, Tokyo, Japan). Flies were then maintained at room temperature (for mRNA analysis) or 29 ℃ (for colony-forming assay) until they were subjected to the analyses. In an assay for colony formation, either bacteria suspension or lysates of *E. coli*-infected flies obtained by homogenization of 5 live flies using a plastic pestle were plated onto agar-solidified Luria-Bertani medium at varying dilutions with phosphate-buffered saline and maintained at 37 ℃ overnight. The number of colonies was then determined, and the colony-forming unit was calculated in a quantitative

manner.

RESULTS

Messenger RNA profile of *E. coli* exposed to *Drosophila* immune proteins

PGRP-LC, a single-path membrane protein, activates the IMD pathway through direct interaction with an adaptor protein called Imd when bound by DAP-type peptidoglycan of Gram-negative bacteria (38) (Fig. 2). There are three subtypes of this receptor, namely, a, x, and y, which are produced by alternative splicing of a single primary transcript and differ in some of their amino acid sequences in the extracellular region (39) (Fig. 3). They form a heterodimer within subtypes as well as other types of PGRP to become active for the binging to peptidoglycan (40,41). Peptidoglycan may undergo partial digestion by the enzyme amidase, the activity of which is exhibited by some types of PGRP, and the heterodimer composed of PGRP-LCa and PGRP-LCx, and the homodimer of PGRP-LCx bind monomeric and polymeric DAP-type peptidoglycan, respectively (40,41) (Fig. 2). I used all three subtypes of PGRP-LC together with the antimicrobial peptide attacin as possible stimulants of gene expression in Gram-negative *E. coli.* The extracellular portions of PGRP-LCa, -LCx, and -LCy (Fig. 3), and full-length attacin were prepared as fusion proteins to GST (Fig. 4), and these PGRP-LC proteins possessed the activity of binding to peptidoglycan of *E. coli* as determined in a solid-phase assay (Fig. 5). *E. coli* that had

grown to the stationary phase of cell growth was incubated in the presence of a mixture of these four proteins, and their RNA was subjected to DNA microarray analysis for mRNA repertoire. As a negative control, RNA of *E. coli* incubated with GST alone was similarly analyzed. When the data from triplicate experiments, a total of 6 groups, were analyzed for hierarchical clustering, they were clearly separated into two clusters, one consisting of 3 groups with the data obtained with *E. coli* exposed to immune proteins and the remaining 3 groups with the data from the control experiment (Fig. 6). This indicated that the mRNA profile significantly differed between *E. coli* incubated with immune proteins and GST. I found 133 and 204 annotated genes whose mRNA increased and decreased, respectively, after incubation with immune proteins. GO analysis of the data revealed that the up-regulated genes were enriched with those coding for proteins involed in the cellular metabolism and stress response (Table 3).

I chose 31 up-regulated genes that showed relatively high scores of increment and coded for proteins involved in sensing environmental conditions (Table 4) and determined which protein, PGRP-LC or attacin, is responsible for an increase in the level of mRNA of those genes. For this purpose, *E. coli* was incubated with a mixture of GST-PGRP-LCa, GST-PGRP-LCx and GST-PGRP-LCy, GST-attacin only, or GST, and their mRNA were analyzed by semi-quantitative reverse transcription-mediated PCR (Fig. 7). I found that 4,

6, and 7 genes were induced by GST-PGRP-LC, GST-attacin, and either protein, respectively, while the level of signals derived from mRNA of the remaining 14 genes did not significantly change under the experimental conditions employed.

Identification of *E. coli nlpI* whose mRNA level increases in adult flies dependently on PGRP-LC

I further characterized the 4 PGRP-LC-inducible genes for validating the hypothesis that a membrane receptor of immune cells stimulates bacteria to alter the pattern of gene expression. I first determined whether those genes were expressed in *E. coli* under the infectious condition. Adult male flies (w^{1118}) were abdominally injected with *E. coli*, a surrogate method to cause sepsis, collected at various time points, and analyzed for the levels of mRNA of *mtfA, nlpI, ybhQ*, and *ydcS* by semi-quantitative reverse transcription-mediated PCR. The data were normalized on the assumption that the level of mRNA of the α-subunit of *E. coli* RNA polymerase encoded by *rpoA* remains unchanged after infection (42). The data indicated that mRNA of *nlpI* exhibited a transient increase 5 to 15 min after injection, while mRNA of the remaining 3 genes gradually decreased (Fig. 8).

I next examined the mode of PGRP-LC-induced expression of lipoprotein-encoding *nlpI* (43) (Fig. 9). A precise time-course was taken to determine at which time point the level of

mRNA of *nlpI* changed, and I found that *nlpI* expression increased 2 to 5 min after infection and returned to the original level by 20 min in w^{1118} flies. PGRP-LC dependence of *nlpI* induction was then examined using $PGRP\text{-}LC^{7454}$, a PGRP-LC-lacking mutant fly line that shows a reduced response to *E. coli* infection in terms of the production of antimicrobial peptides including attacin (22). I found that there was no change in the level of NlpI mRNA in $PGRP\text{-}LC^{7454}$ flies at any time points analyzed. Furthermore, lack of PGRP-LE, a soluble PGRP recognizing *E. coli* either independently from or dependently on PGRP-LC (23,44), did not seem to influence the transient expression of *nlpI*. Finally, an increase of NlpI mRNA was not seen in a fly line that lacked both PGRP-LC and PGRP-LE. These results indicated that PGRP-LC, not PGRP-LE, was responsible for the transient increase of NlpI mRNA in *E. coli* after infection of adult flies.

Involvement of peptidoglycan in PGRP-LC induction of *nlpI* in *E. coli*

It is likely that PGRP-LC triggers *nlpI* expression in *E. coli* by binding to peptidoglycan present in the cell wall. To examine this, I included commercially available *E. coli* peptidoglycan, which had been made partially soluble by sonication, as a competitor in the incubation of *E. coli* with GST-PGRP-LC or GST (Fig. 10). The addition of peptidoglycan had no effect on the expression of *nlpI* in *E. coli* exposed to GST alone. In contrast, the

level of NlpI mRNA-derived signal further increased when *E. coli* was incubated in the presence of GST-PGRP-LC and peptidoglycan. This result, opposite to my expectation, indicated that free peptidoglycan did not serve as a competitive inhibitor for the action of PGRP-LC but rather enhanced it. This effect of peptidoglycan on *nlpI* expression was not observed in the absence of PGRP-LC, suggesting functional interaction between the two molecules. A preferred interpretation of this phenomenon is that supplemented peptidoglycan forms a complex with PGRP-LC, and that this complex stimulates an as-yet unidentified component of *E. coli* to transmit a signal for the induction of *nlpI* expression.

Role for *nlpI* in persistence of *E. coli* in flies

I next attempted to clarify the role for *nlpI* in the behavior of *E. coli* during infection. The basal characteristics of the *nlpI*-deficient mutant (JW3132) and its parental (BW25113) *E. coli* strains were determined when they were maintained in Luria-Bertani medium. These two strains grew almost equally in a liquid medium (Fig. 11 A), and colony-forming efficiency did not differ between them (Fig. 11 B). When a change in the number of colony-formable bacteria in control flies (w^{1118}) infected with the two *E. coli* strains was determined, I found that the NlpI-lacking strain decreased more rapidly than the parental strain (Fig. 12 A). This was not the case when a fly line lacking PGRP-LC was used as the host (Fig. 12 B).

The number of colony-formable *E. coli* increased in the PGRP-LC-lacking flies, and this is most likely due to a defect of PGRP-LC-mediated immune responses. These results indicated a role for *nlpI* in the persistence of *E. coli* in adult flies either by augmenting the growth rate of *E. coli* in the host or by helping *E. coli* evade host immuntiy.

I next asked if the expression of *nlpI* influenced the pathogenic effect of *E. coli* on the survival of infected flies (Fig. 13). However, there was no significant difference in the rate of fly death between infection with *nlpI*-deficient and parental *E. coli*. This indicated that a transient increase of *nlpI* expression made *E. coli* persistent in adult flies with no change of its pathogenicity.

DISCUSSION

In this study, I performed a series of experiments to validate the hypothesis that host immune proteins trigger invading microbes to alter their gene expression pattern simultaneously with the recognition of pathogens to activate innate immune responses. Data from *in vitro* experiments showed that the mRNA repertoire of *E. coli* changed after exposure to either an immune receptor or an antimicrobial peptide. Four *E. coli* genes raised their level of expression when bacteria were incubated in the presence of the extracellular region of PGRP-LC, a membrane-bound receptor of *Drosophila* that recognizes peptidoglycan of Gram-negative bacteria and induces the expression of a variety of genes coding for immunity-related proteins. This was confirmed *in vivo* with one of these up-regulated genes, lipoprotein-encoding *nlpI*: its mRNA transiently increased in *E. coli* injected into the hemocoel of adult flies in a manner dependent on PGRP-LC but not PGRP-LE, a soluble PGRP also recognizing *E. coli* peptidoglycan. These results support my hypothesis and suggest the reciprocal activation of gene expression between host organisms and invading pathogens (Fig. 14). Although the precise mechanism of PGRP-LC action remains unknown, it is suggested that another component besides peptidoglycan, most probably substance(s) residing in the inner membrane of *E. coli*, plays

a role in the transmission of signals provided by PGRP-LC-bound peptidoglycan. One candidate for such a receptor is a sensor kinase of the two-component regulatory system, which recognizes an environmental change and activates a transcription factor called a response regulator by transferring phosphate to a histidine residue.

There are papers reporting the alteration of bacterial gene expression by host immune factors. A protein named apolipophorin contained in the hemolymph of silkworm binds lipoteichoic acid, a cell wall component of Gram-positive bacteria, and inhibited the expression of *hla* and *hlb* that code for hemolysin, a virulent protein of *Staphylococcus aureus* (45,46). Another paper was recently published that describes research conducted with an approach similar to this study (47). They compared the gene expression pattern in *E. coli* before and after the treatment with recombinant human PGRP and found that genes responding to oxidative, thiol, and metal stresses were induced. This, alongside my study, suggests that PGRP gains access to peptidoglycan present as a component of the cell wall of outer membrane-containing *E. coli*. However, the above-mentioned two studies do not indicate the occurrence of `reciprocal` activation of gene expression because silkworm apolipophorin and human PGRP are soluble proteins present in the body fluid, not serving as a membrane-bound immune receptor. As a preceding example of a host signaling ligand that also serves as a ligand for a bacterial receptor to alter gene expression pattern,

adrenaline/noradrenaline binds and activates QseC and QseE (48,49), sensor kinases of the two-component regulatory system, resulting in a change of gene expession in *E. coli* (50). There is more evidence for the existence of `inter-kingdom signaling`, in which the same molecule plays a role as a ligand in both bacteria and host organism, such as quorum sensing autoinducer of bacteria, and opioid, steroid, and growth factor of mammals (51-53).

My data suggest that lipoprotein-encoding *nlpI* is required for the persistence of *E. coli* in adult flies. There are more than 100 lipoproteins in *E. coli*, and they exist at the surface of the inner or outer membrane by inserting lipid portions into the membranes (54). The functions of *E. coli* lipoprotein in terms of bacterial behavior are largely unknown. In mammals, lipoprotein serves as a ligand for pattern-recognition receptors, Toll-like receptor 2 in particular (55). However, bacterial lipoproteins do not seem to be directly recognized by the immune system of insects (56). Previous reports suggested a role for *nlpI* with pathogenic *E. coli* strains: NlpI is required for the adhesion and invasion of the *E. coli* strain LF82 to epithelial cells (57); and NlpI endows *E. coli* O157:H7 strain with resistance to high pressure (58). In *Drosophila*, NlpI could help *E. coli* persist by enhancing the rate of proliferation or mitigating an attack from the host defense. The occurrence of reciprocal activation of gene expression in host and pathogen makes sense in that bacteria begin to brace against host immune response at the same time as their activation.

In the present study, 133 and 204 annotated *E. coli* genes were identified to be up-regulated and down-regulated, respectively, after the treatment with a mixture of PGRP-LC and attacin of *Drosophila in vitro*. Among these genes, only *nlpI* was characterized in details because this was the only gene, among 31 genes chosen from 133 up-regulated genes, whose expression was augmented dependently on PGRP-LC in adult flies. It is therefore necessary to analyze other genes *in vivo*, in particular 13 genes shown to respond to either PGRP-LC or attacin, if expression level rises after infection. In addition, 102 up-regulated genes as well as 204 down-regulated genes remain to be characterized.

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Table 1. The fly lines and *E. coli* strains used in this study.

Fly line	Characteristics
w^{1118}	used as wild type
$PGRP\text{-}LC^{7454}$	lacking PGRP-LC
$PGRP\text{-}LE^{112}$	lacking PGRP-LE
$PGRP\text{-}LE^{112}$; $PGRP\text{-}LC^{7454}$	lacking both PGRP-LE and PGRP-LC

E. coli strain	Characteristics
BW25113	*E. coli* K-12 strain used as parent to JW3132
JW3132	*nlpI*-deficient strain

Table 2. Primers used in reverse transcription-mediated PCR analysis of mRNA.

Gene symbol	Sequence (5'—3')	Expected size of cDNA (bp)	Reference
cspA	F: atgtccggtaaaatgactggt R: ttacaggctggttacgttacc	213	This study
dppA	F: accgtcgcagcaagtgttc R: agctgccgccagaaacttta	358	This study
dsdX	F: accatcgtgaagttcaaattcc R: tacatagcggaatggcaagct	384	This study
exbB	F: attgtcgttaagtgcgtgatg R: cgcgataccaataaagctgtt	420	This study
exbD	F: tggcaatgcatcttaacgaaaa R: ttcgccgaccagacctatct	400	This study
gatA	F: atgactaacctgtttgttcgta R: taacttgccaaataaacagcgt	360	This study
gatB	F: gaaacgcaagattattgtcgc R: caccctgtaagatagtcag	282	This study
glcD	F: atgagcatcttgtacgaagag R: ttatgcggtgcaacggcct	420	This study
mraZ	F: ttatcgggaacagctgcttg R: tagagacaagtcctgcagtc	394	This study
mtfA	F: gattcattaagaagctgccgg R: aatgttgttcattgcagcatg	381	This study
nlpI	F: aggaagtgattctggcacgt R: atcgtcttgataaaacgccag	362	This study
rpoA	F: atgcagggttctgtgacaga R: aacgccttctttggtgctgt	222	Ref. 14
rpoE	F: gcctttaacttactggtagtg R: ggttattgccatgcgtaaatc	380	This study
rseA	F: aacgaactggctcataaccc R: tcggcagtgtattaaataccg	361	This study
rseB	F: atgaagcaactttggtttgcc R: aagacgatcagcaatacgcg	420	This study
yaiY	F: atggctgatttcaccctgtc R: atgtcgccagtgcagccata	297	This study
ybhQ	F: atgaagtggcaacaacgtgtt R: ttcgccagcaaagacactac	362	This study
ydcS	F: atgagcaagacatttgcccg R: ccagtcgcctttaaccacg	398	This study

F, forward; R, reverse.

Table 3. Significantly enriched GO terms in *E. coli* after exposure to immune proteins.

GO-ID	GO term	*p* value
0071840	Cellular component organization or biogenesis	
0016043	Cellular component organization	
0043933	**Macromolecular complex subunit organization**	2.92E-02
0050896	Response to stimulus	
0009628	Response to abiotic stimulus	1.21E-03
0009266	**Response to temperature stimulus**	6.81E-03
0008152	Metabolic process	
0044237	Cellular metabolic process	
0006091	Generation of precursor metabolites and energy	4.53E-02
0009060	Aerobic respiration	1.99E-02
0006099	**Tricarboxylic acid cycle**	2.42E-02
0051187	Cofactor catabolic process	2.64E-02
0009109	Coenzyme catabolic process	2.64E-02
0048356	**Acetyl-CoA catabolic process**	2.42E-02
0006084	Acetyl-CoA metabolic process	3.62E-02
0071704	Organic substance metabolic process	
0016052	**Carbohydrate catabolic process**	2.79E-02
0019751	Polyol metabolic process	5.80E-06
0019400	Alditol metabolic process	5.80E-06
0006059	Hexitol metabolic process	5.35E-05
0019402	**Galactitol metabolic process**	5.35E-05

GO term with no *p* value means 'not significant.' The GO terms appearing in the deepest hierarchy are shown with boldface.

Table 4. List of 31 up-regulated genes subjected to further analysis.

Gene symbol	Characteristic of encoded protein	FDR
cspA	cold shock protein, RNA chaperone and anti-terminator	0
gatA	subunit IIA of galactitol-specific transporter	0
dppA	periplasmic protein, dipeptide transporter	0
gatZ	tagatose 6-phosphate kinase 1	0
nlpD	lipoprotein, activator of murein hydrolase AmiC	0
glcD	subunit of glycolate oxidase	0
yebV	hypothetical protein	0
tnaC	tryptophanase leader peptide	0
iscR	DNA-binding transcriptional regulator	0
rseA	anti-RNA polymerase sigma factor SigE	1.25E-04
rpoS	RNA polymerase sigma S (sigma 38) factor	1.33E-04
dps	DNA starvation/stationary phase protection protein	1.54E-04
gatB	subunit IIB of galactitol-specific transporter	1.67E-04
rpoE	RNA polymerase sigma E (sigma 24) factor	1.90E-04
yaiY	inner membrane protein	2.00E-04
mtfA	anti-repressor for DgsA (Mlc)	3.08E-04
ibpB	heat shock chaperone	3.20E-04
exbB	biopolymer transporter	6.43E-04
ydcS	ABC transporter periplasmic-binding protein	6.67E-04
nlpI	lipoprotein involved in osmotic sensitivity and filamentation	6.88E-04
dmlR	DNA-binding transcriptional activator for *dmlA*	1.06E-03
hslV	subunit of ATP-dependent protease peptidase	2.10E-03
ybhQ	inner membrane protein	3.68E-03
dsdX	D-serine permease	3.73E-03
glnH	subunit of glutamine transporter	6.08E-03
sdhC	membrane subunit of succinate dehydrogenase	6.56E-03
rseB	negative regulator of sigma E	9.51E-03
exbD	biopolymer transporter	1.04E-02
mraZ	inhibitor of methyltransferase RsmH	2.79E-02
ugpB	subunit of glycerol-3-phosphate transporter	3.01E-02
csiE	stationary phase inducible protein	3.07E-02

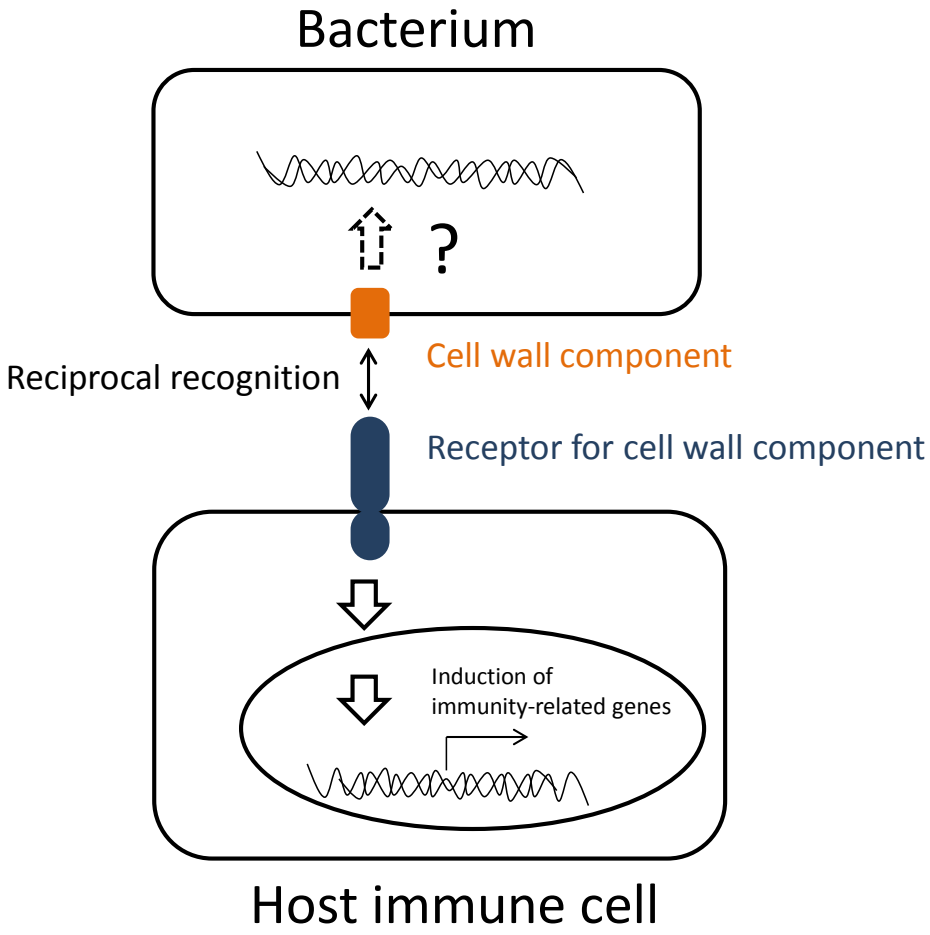

Figure 1. The hypothesis of this study: Reciprocal activation of gene expression between host organisms and pathogens.

Recepters of host immune cell function as ligands to activate cell wall components that serve as receptors for the induction of bacterial genes.

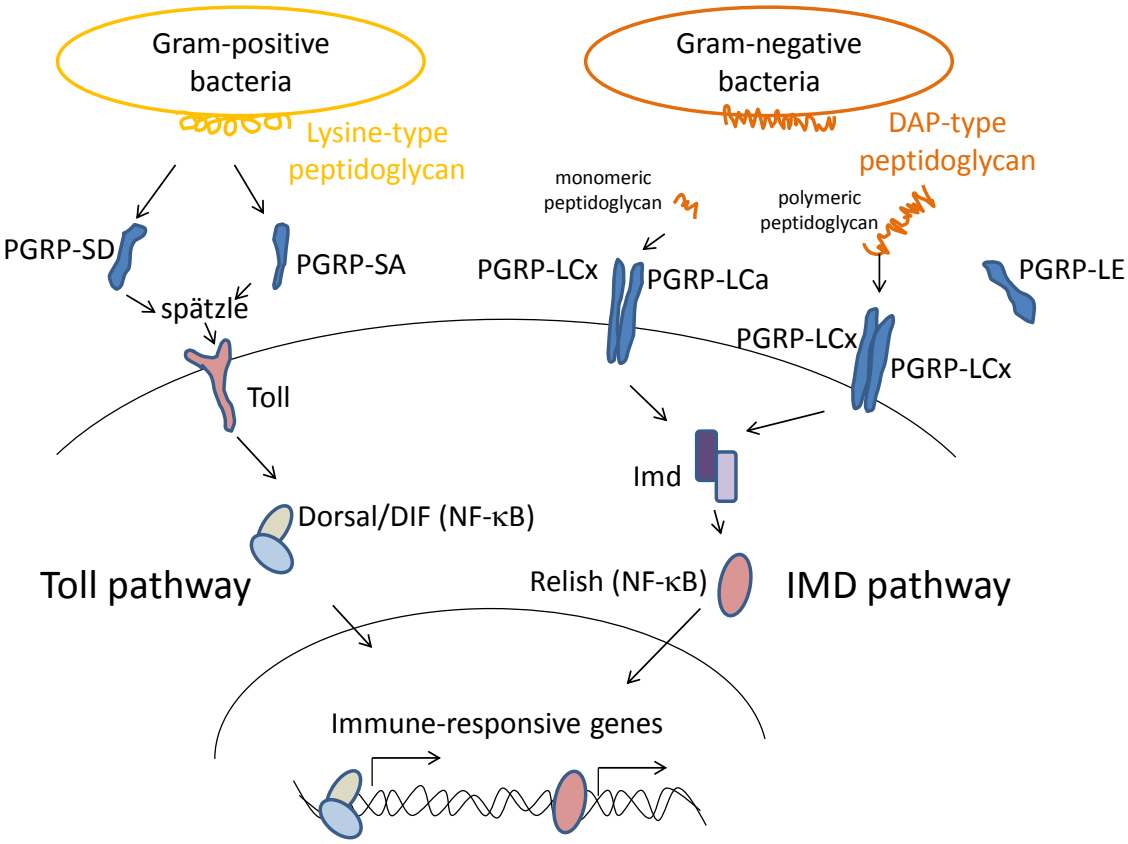

Figure 2. Recognition of bacteria and induction of humoral immune responses in *Drosophila*.

The immune system of *Drosophila* recognizes invading bacteria using peptidoglycan-binding PGRPs that exist either as soluble or membrane-bound forms. Gram-positive and -negative bacteria are differentially recognized by distinct sets of PGRP. Peptidoglycan-bound PGRP activates signaling pathways called Toll and IMD culminating in the NF-κB-mediated expression of a variety of genes that code for proteins responsible for the humoral immune response. Refer to the text for details.

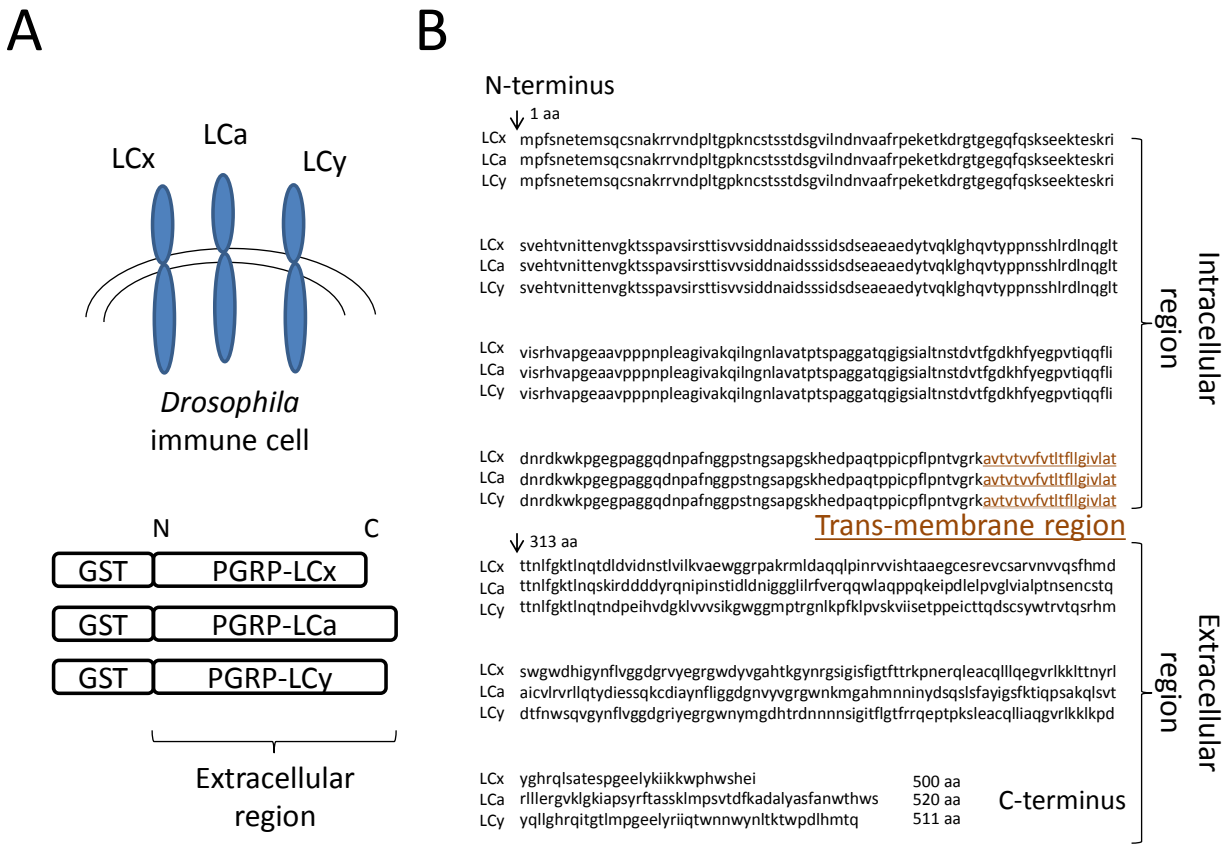

Figure 3. Structure of PGRP-LC.

(A) The topology of 3 subtypes of PGRP-LC and the structure of recombinant GST-fused PGRP-LCs are schematically exhibited. (B) Amino acid sequences of PGRP-LCs are shown with the single-letter notation of amino acids.

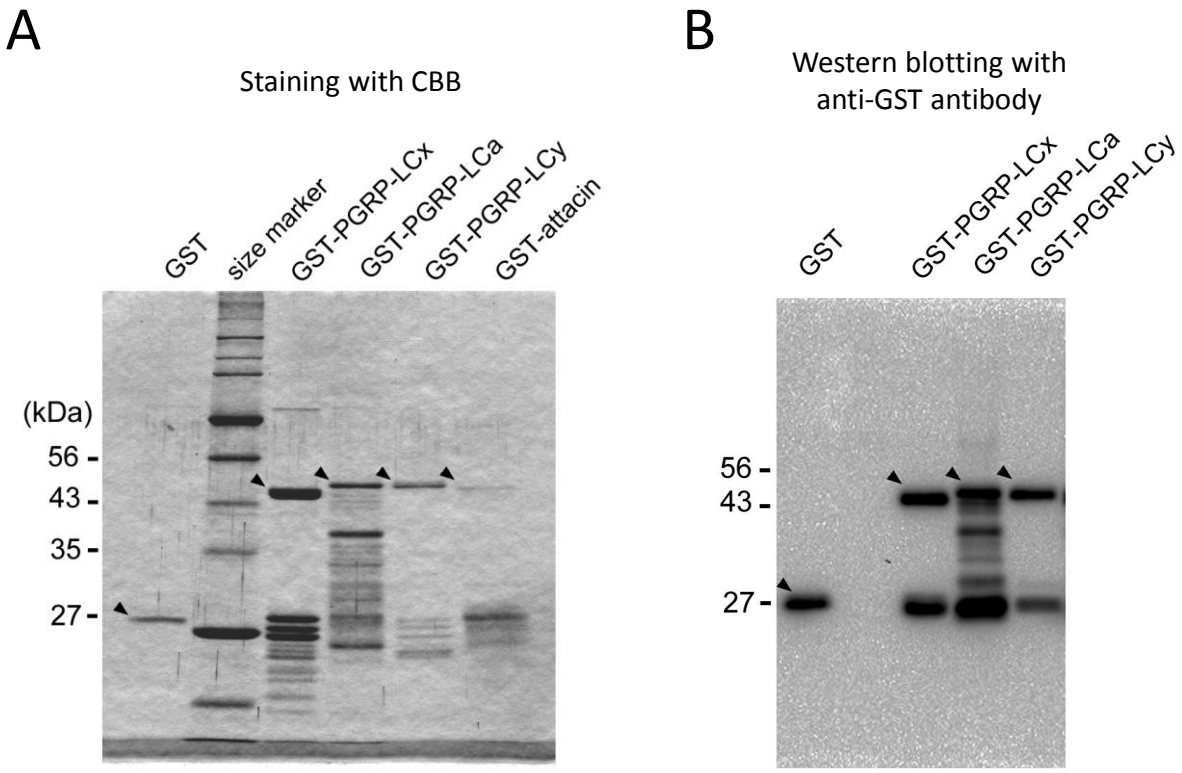

Figure 4. Preparation of GST-PGRP-LCs, GST-attacin and GST.

The extracellular region of 3 subtypes of PGRP-LC and full-length attacin were recombinantly expressed as GST-fusion proteins. These proteins together with GST alone were affinity-purified and analyzed by SDS-PAGE. (A) A Coomassie Brilliant Blue (CBB)-stained gel (0.4~1.4 μg of proteins loaded) is shown. (B) GST-PGRP-LC and GST were subjected to a Western blotting analysis with anti-GST antibody. The arrowheads point to the full-length recombinant proteins.

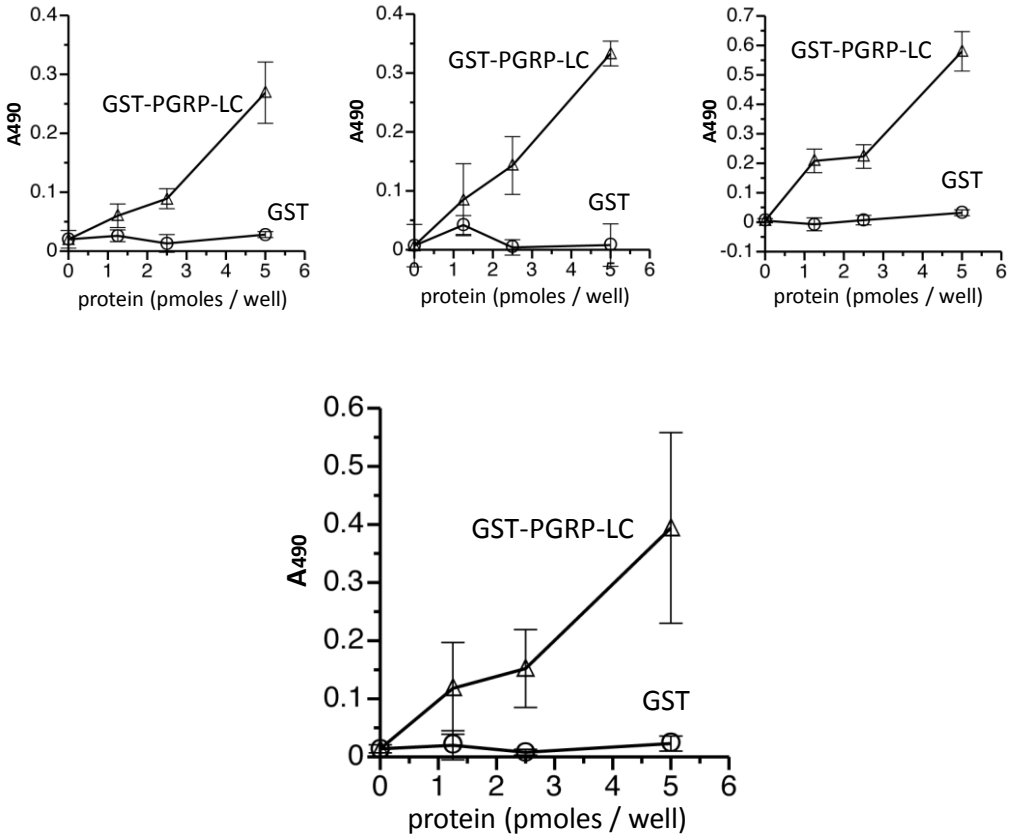

Figure 5. Binding of GST-PGRP-LC to peptidoglycan.

A mixture of 3 GST-PGRP-LCs or GST alone was subjected to a solid-phase assay for binding to peptidoglycan. The same experiment (each dose with 3 wells) was repeated 3 times (3 panels at the top), and those data were combined and shown at the bottom. The data are expressed as the mean ± standard deviation. The amount of proteins is equivalent to GST based on the data from Western blotting (see Figure 4B).

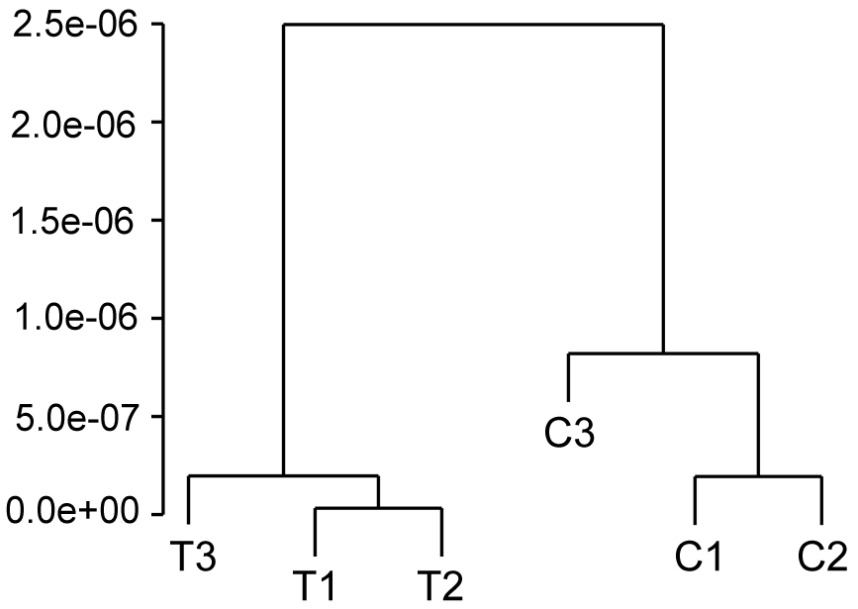

Figure 6. DNA microarray analysis of *E. coli* mRNA after exposure to immune proteins.

E. coli was incubated with a mixture of 3 GST-PGRP-LCs and GST-attacin, or GST alone, and their RNA was subjected to DNA microarray analysis for the repertoire of mRNA. The DNA microarray data with 6 groups, 3 each for incubation with the immune proteins (T1 ~ T3) and GST (C1 ~ C3), were quantified with DFW and exhibited as a hierarchical cluster dendrogram. The vertical scale represents a distance between clusters.

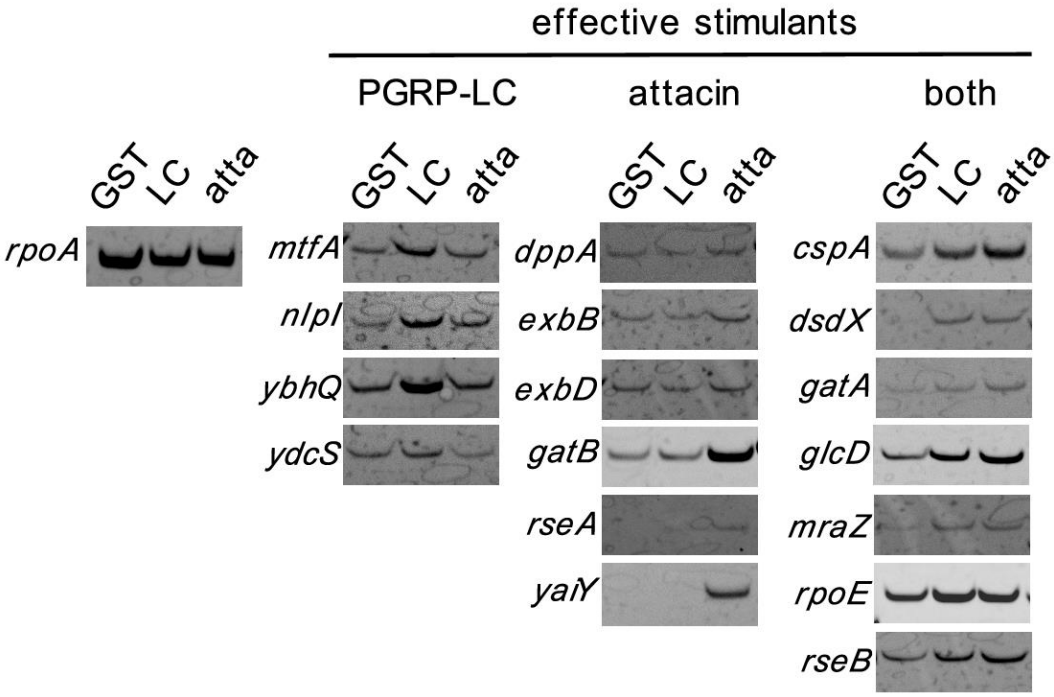

Figure 7. *E. coli* genes induced by PGRP-LC and attacin.

E. coli was incubated with GST- PGRP-LC (x, 1 μM; a, 0.5 μM; y, 0.5 μM) (LC), GST-attacin (0.13 μM) (atta) or GST alone (3 μM) (GST) for 10 min at room temperature, and their RNA was subjected to semi-quantitative reverse transcription-mediated PCR. Thirty-one *E. coli* genes of which mRNA increased in DNA microarray analysis together with control *rpoA* were analyzed. Portions of ethidium bromide-stained gel are shown under classification of effective stimulants. The level of signals derived from mRNA of the remaining 14 genes did not differ before and after incubation with any proteins, and the data are not shown.

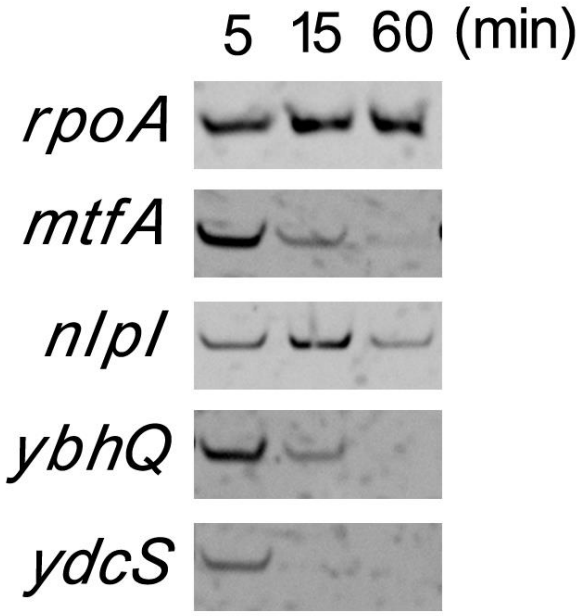

Figure 8. Expression of 4 PGRP-LC-inducible *E. coli* genes in *Drosophila*.

Adult flies (w^{1118}) were abdominally injected with *E. coli*, and RNA was extracted from flies at the indicated time points followed by reverse transcription-mediated PCR. The indicated 4 *E. coli* genes, of which mRNA increased after incubation with GST-PGRP-LC *in vitro* (see Figure 7), together with control *rpoA* were analyzed. Portions of ethidium bromide-stained gel containing the PCR products are shown.

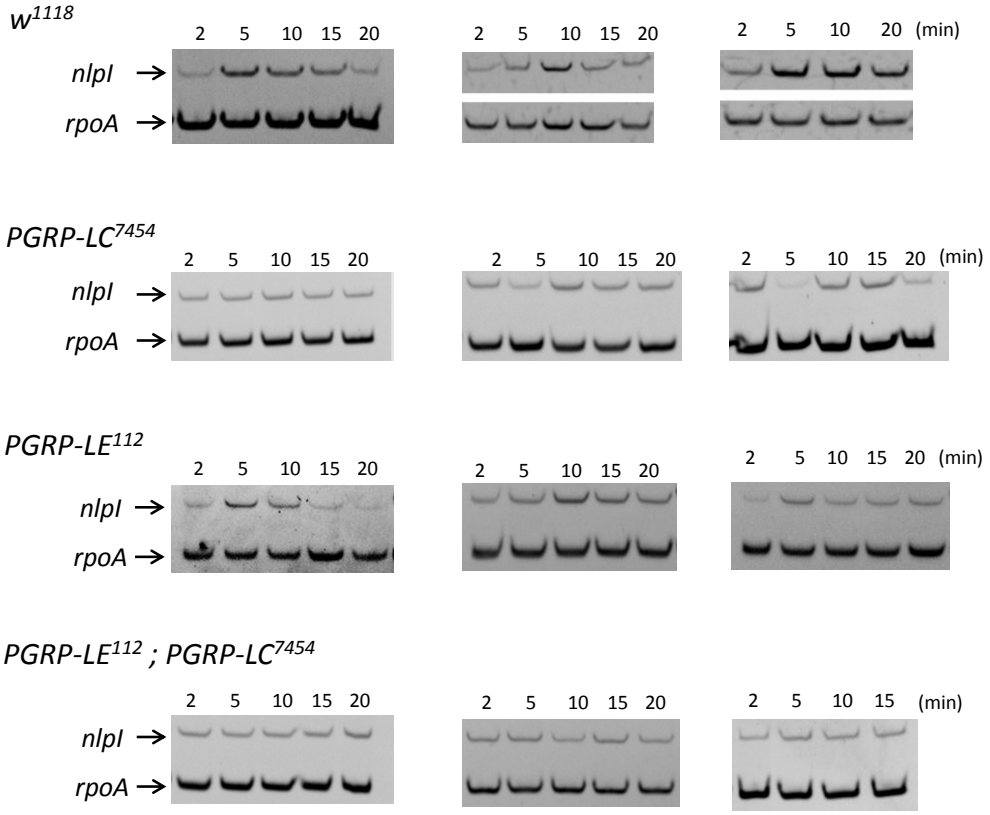

Figure 9. Expression of *nlpI* in wild-type and *PGRP* mutant *Drosophila* lines.

Adult flies of the indicated 4 lines were abdominally injected with *E. coli*, and RNA was extracted at the indicated time points followed by a reverse transcription-mediated PCR analysis of NlpI and RpoA mRNA. Portions of ethidium bromide-stained gel containing the PCR products are shown. The 3 panels shown for each fly line indicate the data from repeated experiments. PCR was carried out with mixed primers for NlpI and RpoA mRNA except for the middle and right panels for w^{1118} where the two mRNA were individually analyzed.

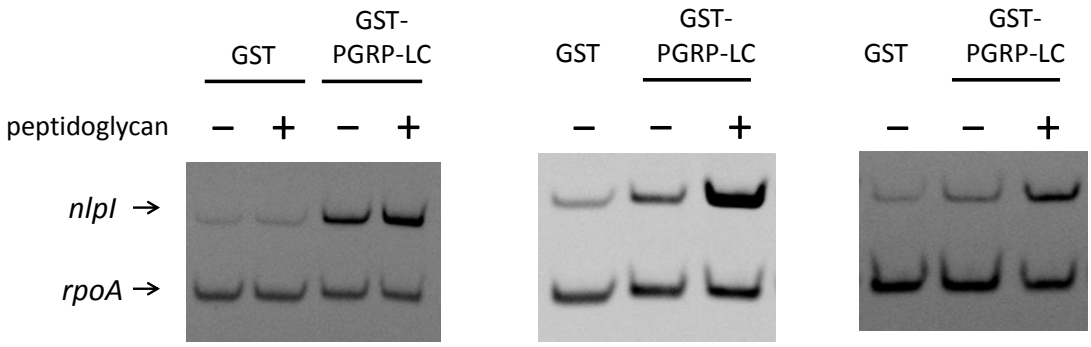

Figure 10. Effect of peptidoglycan on GST-PGRP-LC stimulation of *nlpI* expression.

E. coli was incubated for 10 min at room temperature in the presence of GST-PGRP-LC (a, 0.25 μM; x, 0.5 μM; y, 0.25 μM) or GST alone (1.5 μM) with and without the addition of partially solubilized peptidoglycan (0.44 mg/ml), and subjected to a reverse transcription-mediated PCR analysis of NlpI and RpoA mRNA. Portions of ethidium bromide-stained gel containing the PCR products are shown. The data from 3 independent experiments are presented.

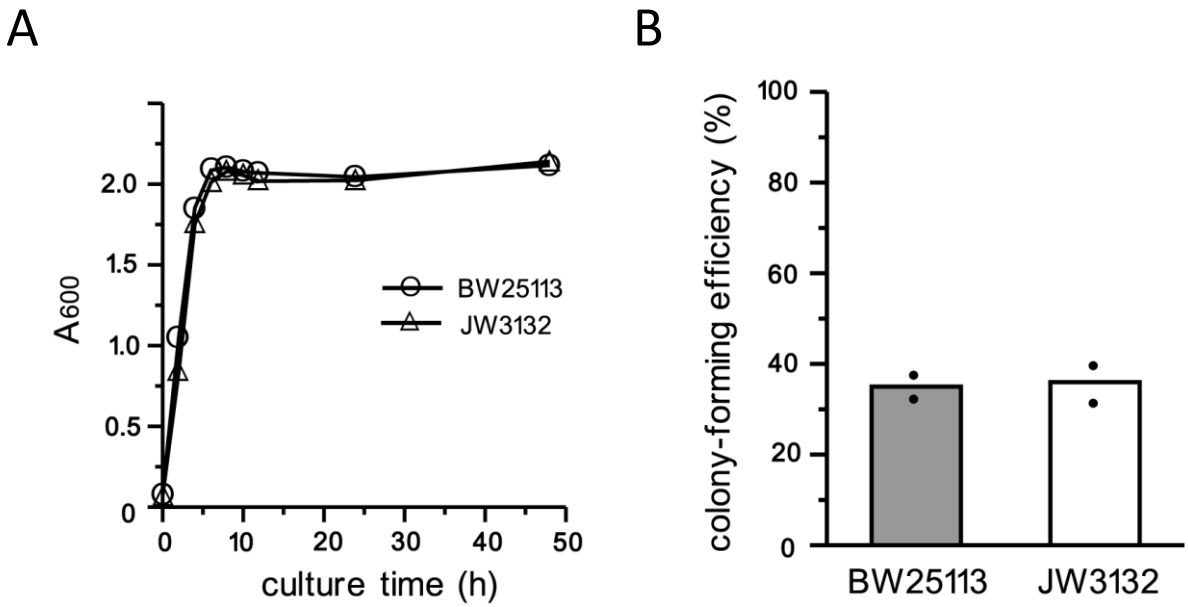

Figure 11. Basal characteristics of *nlpI*-deficient *E. coli*.

E. coli strain lacking NlpI (JW3132) and its parental (BW25113) were analyzed for the growth rate (A) and colony-forming ability (B) maintained in Luria-Bertani medium. Colony-forming ability was determined as a ratio of the number of colonies to that of bacterial cells and is shown in percentage terms with values obtained in two independent experiments.

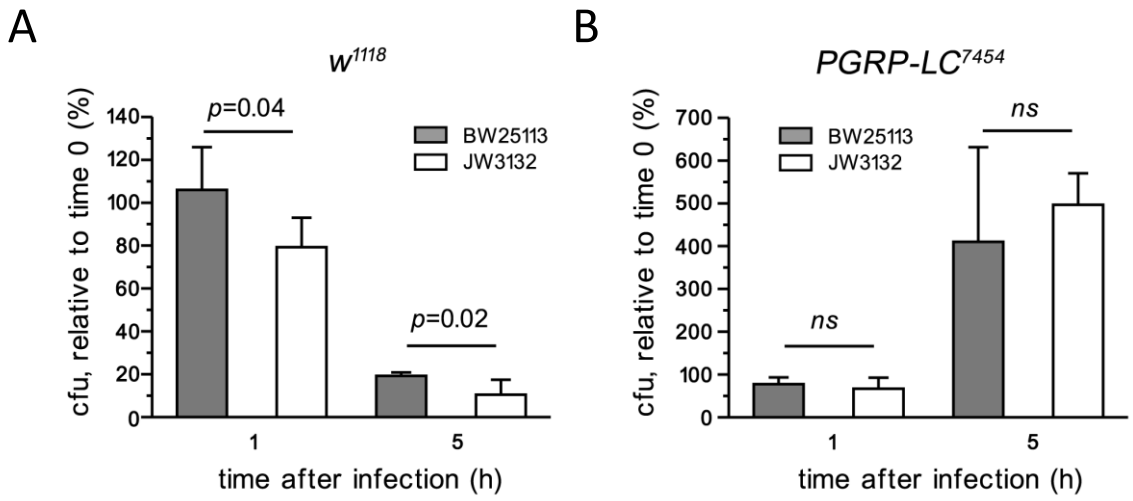

Figure 12. Effect of *nlpI* on persistence of *E. coli* in *Drosophila*.

Adult flies of w^{1118} (A) and $PGRP\text{-}LC^{7454}$ (B) were abdominally injected with *E. coli* stains JW3132 (*nlpI* mutant) and BW25113 (parent). The flies were collected after 0 (immediately after injection), 1 and 5 h, and the lysates prepared from 5 live flies were analyzed for the level of colony-formable bacteria. Colony-forming unit (cfu) was determined, and the cfu at 1 and 5 h relative to at 0 h is shown in percentage terms. The experiments were repeated 5 times with w^{1118} and 4 (1 h) and 3 times (5 h) with $PGRP\text{-}LC^{7454}$, and the data were statistically analyzed by the two-tailed student`s *t* test and are expressed as the mean and standard deviation. *p* values less than 0.05 were considered significantly different and are indicated in the figures. *ns*, difference not significant.

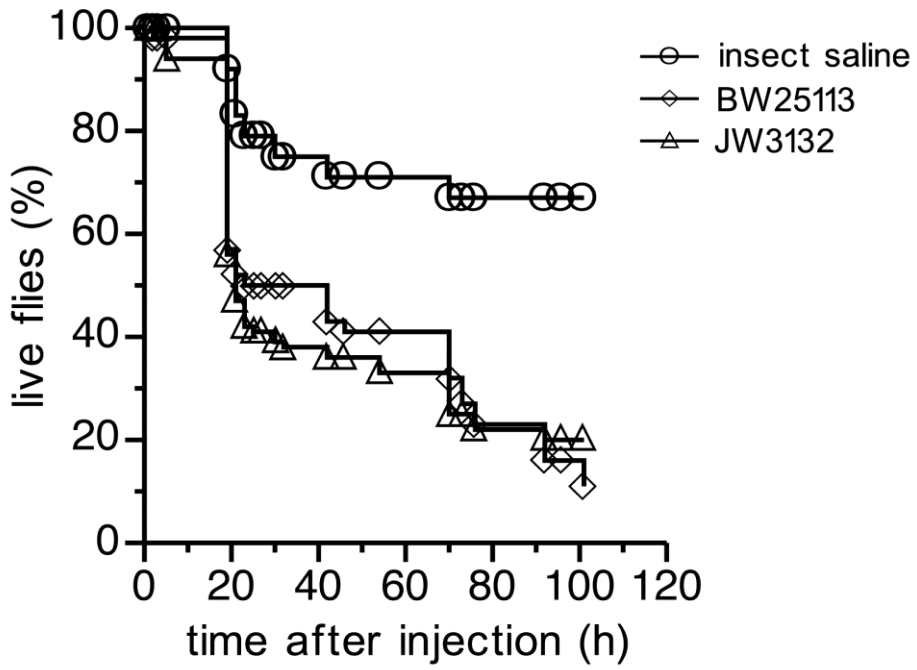

Figure 13. Effect of *nlpI* on pathogenicity of *E. coli* in *Drosophila*.

Adult flies of w^{1118} were abdominally injected with the indicated *E. coli* strains or vehicle (insect saline) alone, and examined for the ratio of live flies at the indicated time points. The numbers of flies used were 33 and 31 (two groups: mean values are shown) for JW3132, 44 for BW25113, and 24 for insect saline.

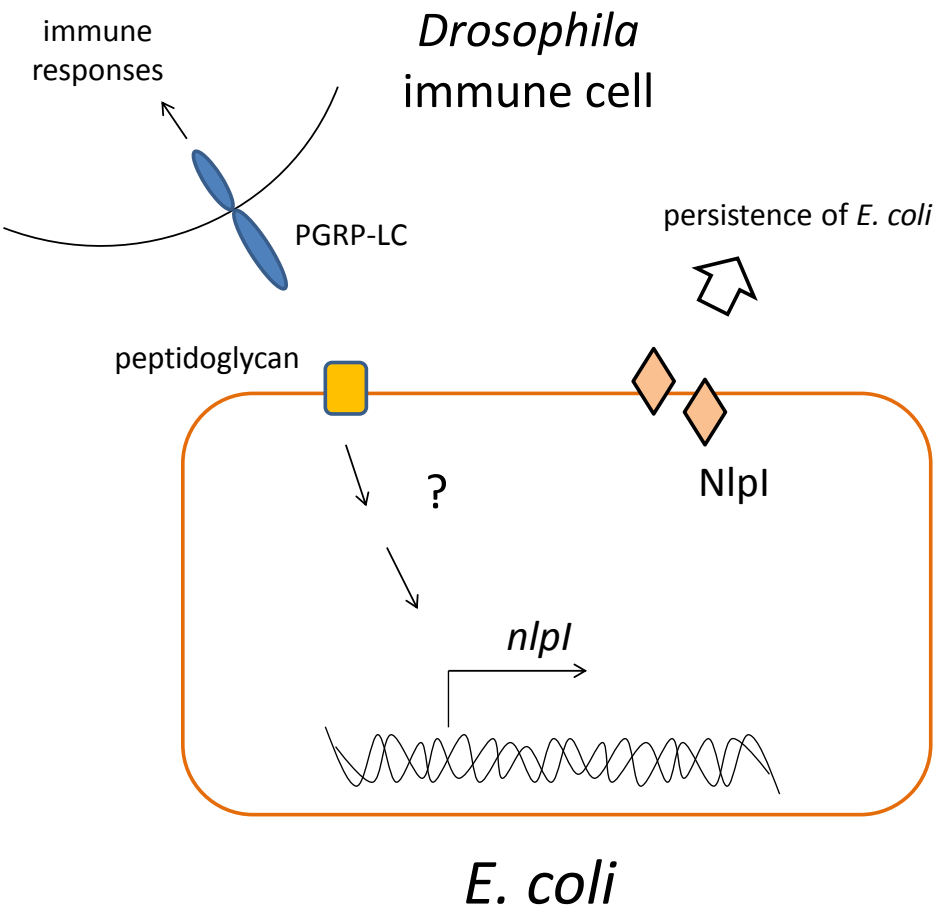

Figure 14. Summary figure: Reciprocal stimulation of gene expression between host immune cells and bacteria, and NlpI-mediated persistence of *E. coli* in *Drosophila*.

PGRP-LC is a membrane-bound receptor of *Drosophila* responsible for immune responses against invading Gram-negative bacteria. This receptor activates host cells to induce immunity-related genes when bound by peptidoglycan of *E. coli*. My study showed that the same receptor simultaneously triggers *E. coli* to alter the pattern of gene expression. Products of the up-regulated genes are likely to be beneficial to *E. coli*, as exemplified by the lipoprotein NlpI that makes *E. coli* persistent in adult flies. Precise mechanisms for the signaling pathway located downstream of peptidoglycan remain to be known.