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Peptidoglycan recognition protein-triggered induction of *Escherichia coli* gene in *Drosophila melanogaster*

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Running title: PGRP-triggered change of bacterial gene expression

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Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeat; DAP, *meso*-diaminopimelic acid; DEGs, differentially expressed genes; DFW, Distribution Free Weighted method; FDR, false discovery rate; GO, Gene Ontology; GST, GSH *S*-transferase; MIAME, Minimum Information About a Microarray Experiment; NF, nuclear factor; PGRP, peptidoglycan recognition protein(s).

Summary

Interaction between the host and pathogen determines the fate of both organisms during the infectious state. The host is equipped with a battery 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. We 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*, and subjected to DNA microarray analysis for mRNA repertoire. We identified 133 annotated genes whose mRNA increased after the treatment, and at least four of them were induced in response to PGRP-LC. One such gene, lipoprotein-encoding *nlpI*, showed a transient increase of mRNA in adult flies depending on PGRP-LC but not PGRP-LE. 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 simultaneously with their recognition and induction of immune responses.

Key words: bacteria/*Drosophila*/gene regulation/immunology cellular response/transcription regulation

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.

We 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 consisting of the cell wall, which evokes the induction of an array of immune reactions including the production of antimicrobial substances and 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. We 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.

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*, 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 bacilli (*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*).

To validate our hypothesis, we took advantage of *Drosophila*, a model animal simpler than mammals with regard to the study of 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 (*nlpI*-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).

Preparation of 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 GSH *S*-transferase (GST) at the N-terminus. 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 GSH-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, 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 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 IgG antibody (GE Healthcare Japan).

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 (0.13 M NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) (*26*) 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 bp 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 °C 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 Microarray Gene Expression Data Society (http://www.mged.org/Workgroups/MIAME/miame.html).

The original CEL files were quantified with the Distribution Free Weighted method (DFW) (*27*) using statistical language R (http://www.r-project) (*28*) and Bioconductor (http://www.bioconductor.org/) (*29*). Hierarchical clustering was performed using the pvclust() function (*30*) in R. To identify differentially expressed genes (DEGs), the rank products method (*31*) 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/) (*32*) and Quick GO (http://www.ebi.ac.uk/QuickGO/) (*33*). Expression Analysis Systematic Explorer scores, which are modified Fisher's exact test *p* values (*34*), were used to extract statistically overrepresented Gene

Ontology (GO) terms from the DEGs. GO terms with *p* values of <0.05 were regarded as significantly enriched.

Semi-quantitative reverse transcription-mediated PCR

Total 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 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 I. The amplified DNA was separated by 6% 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 the data for other mRNA were exhibited after normalization (*14*).

Infection of adult flies with bacteria and colony-forming assay

E. coli was cultured at 37 ˚C 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 (*35*) with modification (*36*). 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 ˚C (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 ˚C overnight. The number of colonies was then determined, and cfu was calculated in a quantitative manner.

Assay for binding of GST-fused proteins to peptidoglycan

A solid-phase binding assay was conducted essentially as described previously (*37*). In brief, dishes of a

96-well culture container 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-LCa, GST-PGRP-LCx 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 *o*-phenylenediamine as a substrate, and the amount of the reaction products was determined by measuring A_{490}.

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*). 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*). They form a heterodimer within subtypes as well as other types of PGRP to become active for the binding 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*). We used all three subtypes of PGRP-LC as possible stimulants of gene expression in Gram-negative *E. coli*. The antimicrobial peptide attacin, which is the largest among antimicrobial peptides produced in *Drosophila* upon infection with Gram-negative bacteria, was included in the stimulants because this was successfully expressed as a recombinant protein in *E. coli*. The extracellular portions of PGRP-LCa, -LCx, and -LCy, and full-length attacin were prepared as fusion proteins to GST (Fig. 1A), and these PGRP-LC proteins possessed the activity of binding to peptidoglycan of *E. coli* as determined in a solid-phase assay (Fig. 1B). *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. 1C). This indicated that the mRNA profile significantly differed between *E. coli* incubated with immune proteins and GST. We found 133 and 204 annotated genes for which the 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 involved in the cellular metabolism and stress response (Table II and Fig. 2).

We chose 31 up-regulated genes that showed relatively high scores of increment and coded for proteins involved in sensing environmental conditions (Table III) 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 alone, and their mRNA were analyzed by semi-quantitative reverse transcription-mediated PCR (Fig. 3). We 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 (data not shown). It remains to be tested if these 14 genes are induced when bacteria are simultaneously treated with GST-PGRP-LC and GST-attacin.

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

We further characterized the 4 PGRP-LC-inducible genes for validating our hypothesis that a membrane receptor of immune cells stimulates bacteria to alter the pattern of gene expression, while the attacin-induced 6 *E. coli* genes were left for future analysis. We first determined whether those genes were expressed in *E. coli* under the infectious state. 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 (Fig. 4A). 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. We next examined the mode of PGRP-LC-induced expression of lipoprotein-encoding *nlpI* (*43*).

A precise time-course was taken to determine at which time point the level of mRNA of *nlpI* changed (Fig. 4B). We found that *nlpI* expression was raised 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 with a P-element insertion that shows a reduced response to *E. coli* infection in terms of the production of antimicrobial peptides including attacin (*22*). We observed no change in the level of NlpI mRNA at any time points analyzed. In contrast, lack of PGRP-LE (LE^{112}), 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, we 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. 5). The addition of peptidoglycan had no effect on the expression of *nlpI* in *E. coli* exposed to GST alone. In contrast, the level of the NlpI mRNA-derived signal further increased when *E. coli* was incubated in the presence of GST-PGRP-LC and peptidoglycan. This result, opposite to our 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 the functional interaction between the two molecules. Our 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*

We next attempted to identify 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. 6A), and colony-forming efficiency did not differ between them (Fig. 6B). When a change in the number of colony-forming bacteria in control flies (w^{1118}) infected with the two *E. coli* strains was determined, we found that the NlpI-lacking strain decreased more rapidly than the parental strain (left panel in Fig. 6C). This was not the case when a fly line lacking PGRP-LC was used as the host (right panel in Fig. 6C): note that bacteria seemed to grow in flies, most likely due to a defect in 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 immunity.

In summary, the above-described results suggest the existence of mutual control of gene expression between the host and pathogen, that is, the membrane-bound receptor PGRP-LC of immune cells and its ligand peptidoglycan of *E. coli* reciprocally act as a receptor and a ligand to alter the pattern of gene expression in both cell types.

Discussion

In the present study, we performed a series of experiments to validate our 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. An *in vitro* experiment showed that 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 with the extracellular region of *Drosophila* PGRP-LC, a membrane-bound receptor that recognizes peptidoglycan of Gram-negative bacteria and induces the expression of a variety of genes in tissues such as fat body, hemocytes and midgut. 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 our hypothesis and suggest the reciprocal activation of gene expression between host organisms and invading pathogens. Although the precise mechanism of PGRP-LC action remains unknown, our data suggest the presence of another component besides peptidoglycan, most probably on the inner membrane of *E. coli*, which 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 (*7*, *8*).

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*). While this manuscript was under preparation, a paper was published that describes research conducted with an approach similar to ours (*47*). This other study 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 our 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 the 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 expression 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, such as quorum sensing autoinducer of bacteria, and opioid, steroid, and growth factor of mammals (*51–53*).

Our 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 responses at the same time as their activation.

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Conflict of Interest

None declared.

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Figure Legends

Fig. 1 Preparation of *Drosophila* immune proteins, peptidoglycan binding of PGRP-LC, and DNA microarray analysis of *E. coli* mRNA after exposure to immune proteins. (**A**) GST-fused extracellular region of PGRP-LC (a, x, and y) and attacin together with GST alone were expressed in insect cells or *E. coli*, affinity-purified, and analyzed by SDS-PAGE. A Coomassie Brilliant Blue-stained gel (0.4~1.4 μg of proteins loaded) (left) and data from Western blotting analysis with anti-GST antibody (right) are shown with full-length recombinant proteins indicated by arrowheads. (**B**) A mixture of GST-PGRP-LCa, GST-PGRP-LCx and GST-PGRP-LCy (GST-PGRP-LC), or GST was examined for the binding to peptidoglycan of *E. coli* in a solid-phase assay. Protein concentration is equivalent to GST based on the data from Western blotting analysis. The data from 3 independent experiments were statistically analyzed by the two-tailed Student's *t* test and are expressed as the mean ± SD. (**C**) *E. coli* was incubated with a mixture of GST-PGRP-LCa, GST-PGRP-LCx, GST-PGRP-LCy and GST-attacin, or GST alone as a negative control, 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 (T1~3) and GST (C1~3), were quantified with DFW and exhibited as a hierarchical cluster dendrogram. The vertical scale represents a distance between clusters.

Fig. 2 Genes categorized into GO terms enriched in *E. coli* after incubation with *Drosophila* immune proteins. *E. coli* genes (represented as gene symbols) included in the GO terms shown in Table II are exhibited as a Venn and Euler diagram to indicate the interdependency of categories that share DEGs.

Fig. 3 *E. coli* genes induced by PGRP-LC and attacin. *E. coli* was incubated with GST-PGRP-LC (a, 0.5 μM; x, 1 μM; y, 0.5 μM), GST-attacin (0.13 μM) or GST alone (3 μM) for 10 min, 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 (shown in Table III) together with control *rpoA* were analyzed. Portions of ethidium bromide-stained gel are shown under classification of effective stimulants. The panel at the furthest left shows the RpoA mRNA-derived signal with two different amounts of the PCR product

loaded on a gel. 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. An increase in the level of a signal derived from NlpI mRNA was observed with two different preparations of GST-PGRP-LC.

Fig. 4 PGRP-LC dependence of *nlpI* induction in *Drosophila*. The level of mRNA-derived signals of *E. coli* genes was determined by reverse transcription-mediated PCR with RNA prepared from *E. coli*-infected adult flies. Portions of ethidium bromide-stained gel containing the PCR products are shown. (**A**) Control w^{1118} flies were abdominally infected with *E. coli*, and RNA was extracted from flies at the indicated time points followed by reverse transcription-mediated PCR analysis of the 4 PGRP-LC-inducible *E. coli* genes (see Fig. 3) together with control *rpoA*. (**B**) Adult flies of the indicated fly lines were infected with *E. coli*, and RNA prepared from those flies at various time points was analyzed for the level of signals derived from mRNA of *nlpI* and *rpoA*. PCR was carried out with a mixture of the primers for the two target genes. LC^{7454}, $PGRP\text{-}LC^{7454}$ lacking PGRP-LC; LE^{112}, $PGRP\text{-}LE^{112}$ lacking PGRP-LE; LE^{112} ; LC^{7454}, $PGRP\text{-}LE^{112}$; $PGRP\text{-}LC^{7454}$ lacking both PGRP-LC and PGRP-LE. Data from one of 3 independent experiments that gave similar results are shown.

Fig. 5 Enhancement of PGRP-LC-induced expression of *nlpI in vitro* by peptidoglycan. Partially soluble peptidoglycan (PGN) (0.44 mg/ml) was added to *E. coli* suspension simultaneously with GST-PGRP-LC (a, 0.25 μM; x, 0.5 μM; y, 0.25 μM) or GST alone (1.5 μM), and their RNA was analyzed by reverse transcription-mediated PCR for the level of signals derived from mRNA of *nlpI* and *rpoA*. PCR was carried out with a mixture of the primers for the two target genes. A portion of ethidium bromide-stained gel is shown. Representative data from one of 4 independent experiments that yielded similar results is exhibited.

Fig. 6 Comparison of *nlpI*-deficient and parental strains of *E. coli*. NlpI-lacking (JW3132) and its parental (BW25113) *E. coli* strains were compared for growth and persistence. (**A**) The two strains were cultured with Luria-Bertani medium, and the level of growth was determined at the indicated time points by measuring optical density at 600 nm. (**B**) The two strains were cultured with Luria-Bertani medium,

harvested at the stationary phase of cell growth, and subjected to an assay for colony formation. Colony-forming efficiency is shown as a ratio of the number of colonies to that of bacterial cells in percentage terms. The values obtained in two independent experiments are shown with the mean. (**C**) w^{1118} and *PGRP-LC*^{7454} (*LC*^{7454}) flies were abdominally infected with the two strains, collected at the indicated time points, and subjected to a colony-forming assay. The ratio of cfu at each time point to that obtained with flies that had been disrupted immediately after bacterial injection is shown in percentage terms. The data were statistically analyzed by the two-tailed Student's *t* test and are expressed as the mean ± SD of the results from 5 (1 h and 5 h with w^{1118}), 4 (1 h with *PGRP-LC*^{7454}), and 3 (5 h with *PGRP-LC*^{7454}) independent experiments. Any *P* values of less than 0.05 were considered significant and are indicated. *ns*, difference not significant.

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

F, forward; R, reverse.

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

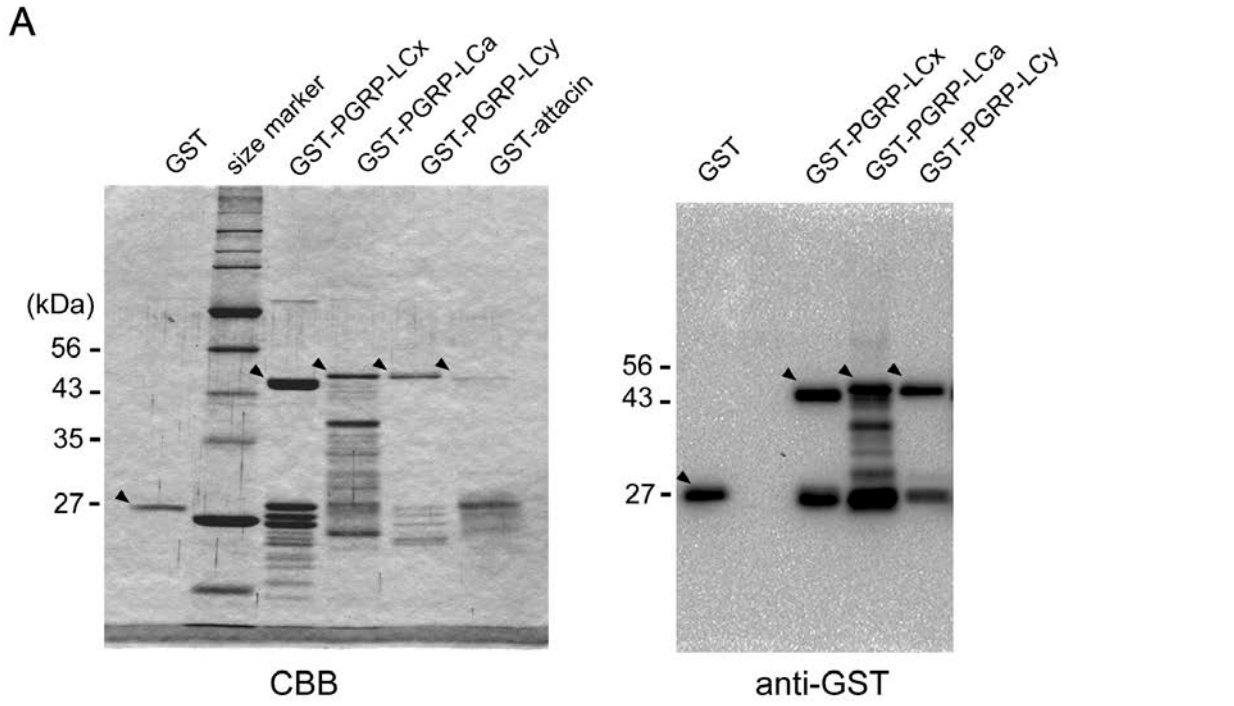
Table II. Significantly enriched GO terms in *E. coli* after exposure to immune proteins.

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

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

Table III. 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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size marker
GST-PGRP-LCx
GST-PGRP-LCa
GST-PGRP-LCy
GST-attacin
(kDa)
56
43
35
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CBB
GST
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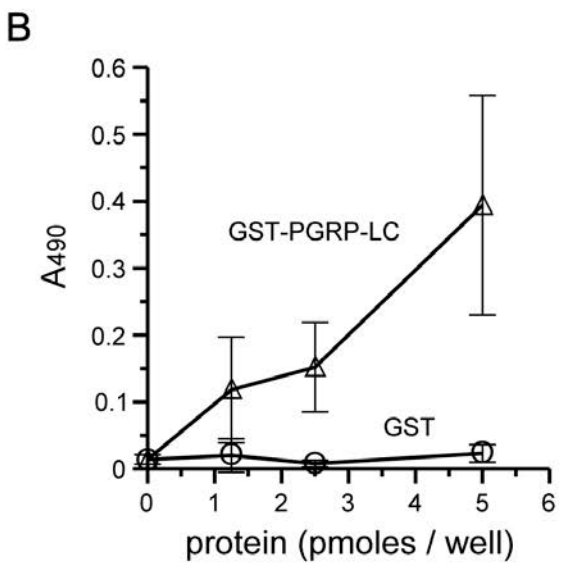
GST-PGRP-LCy
56
43
27
anti-GST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0.5
0.4
0.3
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0.1
0
A490
GST-PGRP-LC
GST
0
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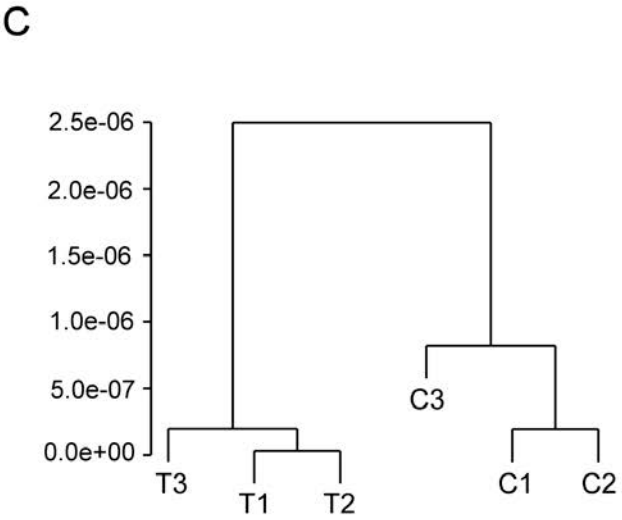
4
5
6
protein (pmoles / well)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2.0e-06
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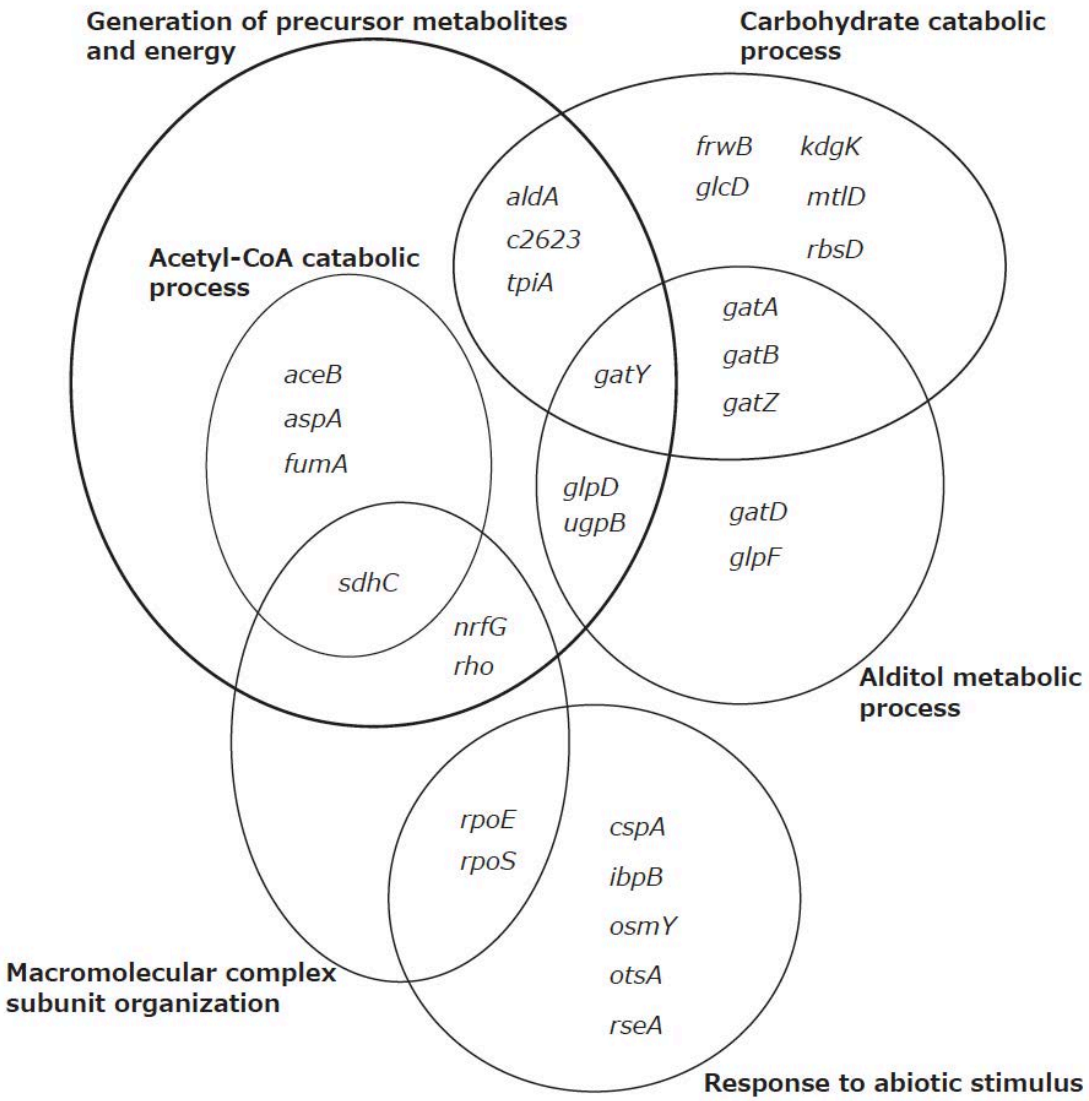
C3
T3
T1
T2
C1
C2


Generation of precursor metabolites and energy
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kdgK
glcD
mtlD
aldA
c2623
tpiA
rbsD
Acetyl-CoA catabolic process
gatA
gatB
gatZ
gatY
aceB
aspA
fumA
glpD
ugpB
gatD
glpF
sdhC
nrfG
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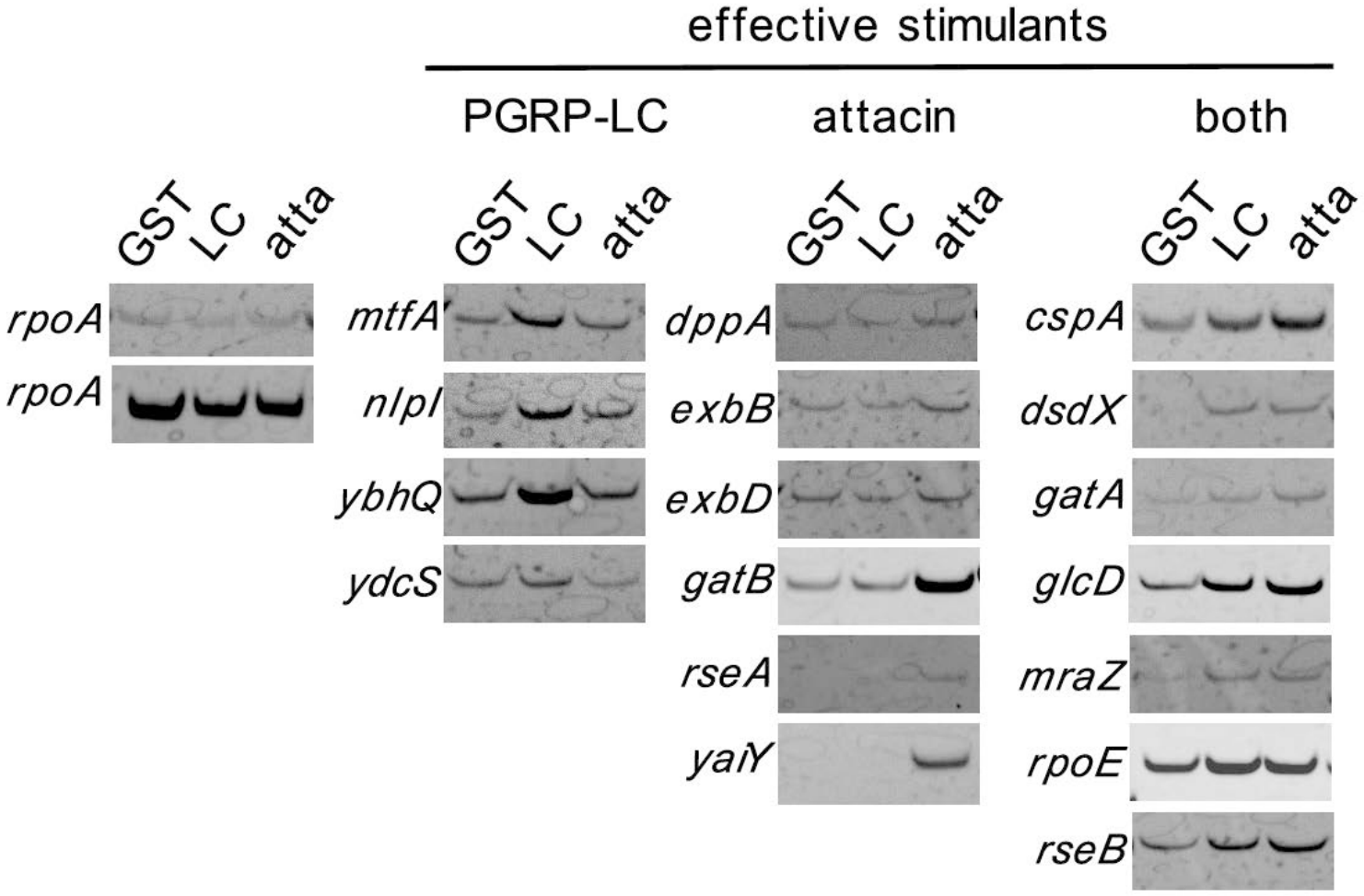
Macromolecular complex subunit organization
Response to abiotic stimulus


effective stimulants
PGRP-LC
attacin
both
GST
LC
atta
rpoA
rpoA
mtfA
nlpI
ybhQ
ydcS
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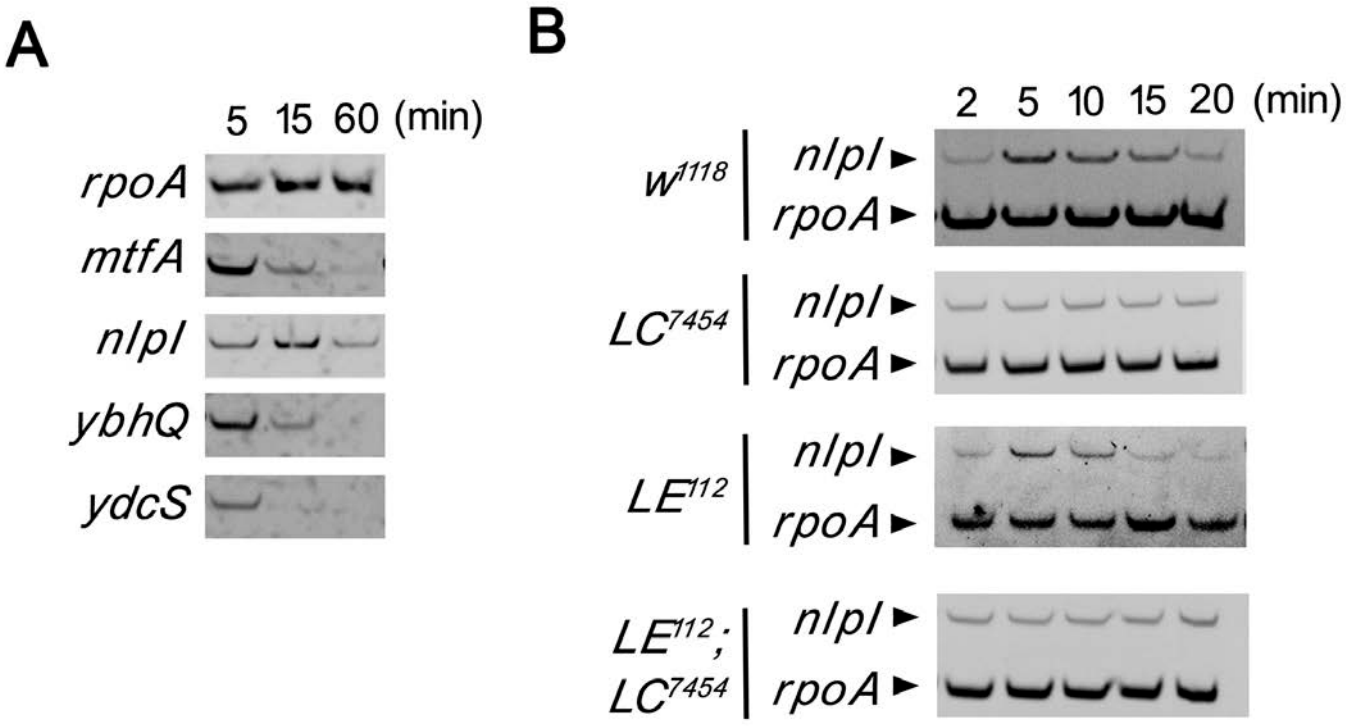
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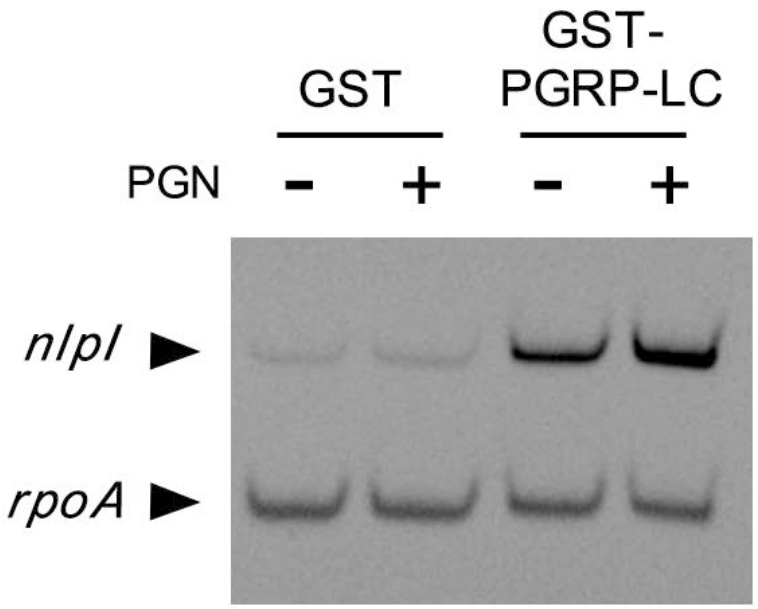
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PGN
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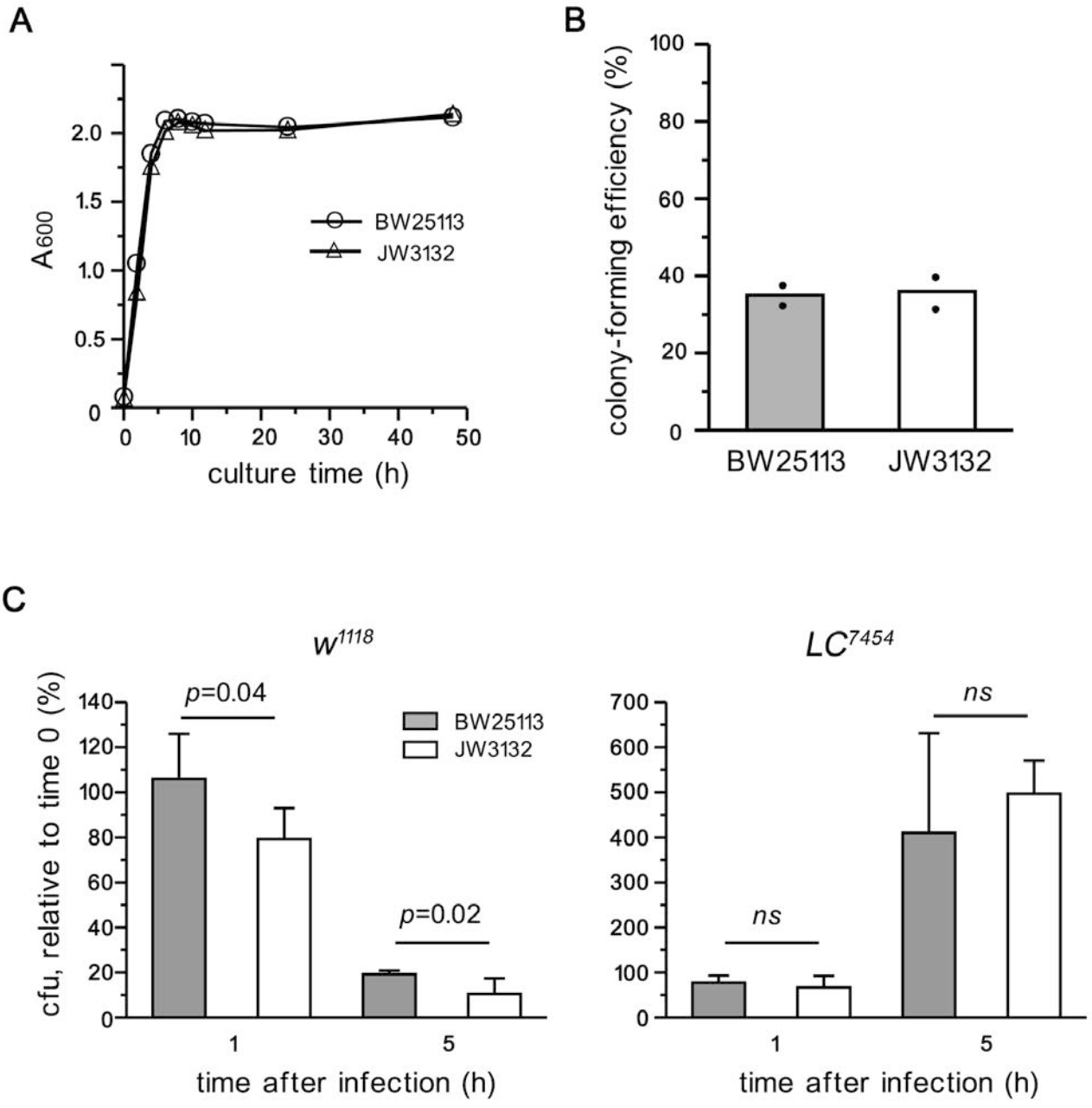
A
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culture time (h)
BW25113
JW3132
B
colony-forming efficiency (%)
BW25113
JW3132
C
w1118
LC7454
cfu, relative to time 0 (%)
p=0.04
p=0.02
ns
ns
BW25113
JW3132
time after infection (h)
time after infection (h)