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# Lipoteichoic Acid and Lipopolysaccharide can Activate Antimicrobial Peptide Expression in the Tobacco Hornworm *Manduca sexta*

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## Abstract

Activation of prophenoloxidase and synthesis of antimicrobial peptides (AMPs) are two important innate immune mechanisms in insects. In the current study, we investigated immune responses activated by three major bacterial components, lipopolysaccharide (LPS) (including rough mutants of LPS), lipoteichoic acid (LTA), and peptidoglycan (PG), in the larvae of a lepidopteran insect, *Manduca sexta*. We found that two DAP (Diaminopimelic acid)-type PGs from *Escherichia coli* and *Bacillus subtilis* were much more potent than LPS and LTA from the respective bacteria as well as a Lysine-type PG in activation of prophenoloxidase in *M. sexta* larval plasma *in vitro*. Transcription levels of AMP genes, such as Attacin, Lebocin and Moricin genes, in the hemocytes and fat body of larvae were significantly induced by smooth LPS (TLR4grade) and rough mutants of LPS (TLRgrade™), synthetic lipid A, LTA, and PG. LPS from *E. coli* and LTA from *B. subtilis* activated AMP expression to significantly higher levels than PGs from the respective bacterial strains, and smooth LPS were more potent than lipid A and rough mutants of LPS in activation of AMP expression. Our results demonstrated for the first time that LTA can activate AMP expression, and different moieties of LPS may synergistically activate AMP expression in *M. sexta*.

### Keywords

Lipopolysaccharide; Lipoteichoic acid; Peptidoglycan; Phenoloxidase; Antimicrobial peptide; *Manduca sexta*

## 1. Introduction

The innate immune system is important to both vertebrates (Mogensen, 2009) and invertebrates (Iwanaga and Lee, 2005), and it relies on a repertoire of germline-encoded pattern recognition receptors (PRRs) to recognize specific pathogen-associated molecular patterns (PAMPs) to initiate cellular and humoral immune responses (Akira et al., 2006; Janeway and Medzhitov, 2002; Kumar et al., 2009). PAMPs are unique molecular patterns

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found only in pathogens but not in host cells, such as bacterial lipopolysaccharide (LPS), peptidoglycan (PG), lipoteichoic acid (LTA), flagellin, unmethylated CpG DNA, and viral dsRNA (Janeway and Medzhitov, 2002; Mogensen, 2009). PRRs include Toll-like receptors (TLRs) (Armant and Fenton, 2002), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) (Kawai and Akira, 2009), peptidoglycan recognition proteins (PGRPs) (Royet and Dziarski, 2007), and C-type lectin like proteins (Hollmig et al., 2009).

The innate immune system of invertebrates share similarities with that of vertebrates, and it also has distinct mechanisms (Kanost et al., 2004; Kimbrell and Beutler, 2001; Kurata et al., 2006; Lemaitre and Hoffmann, 2007). In insects, cellular immune responses include hemocyte-mediated phagocytosis, nodule formation, encapsulation and melanization, whereas activation of the prophenoloxidase (proPO) system and synthesis of antimicrobial peptides (AMPs) are two important humoral defense mechanisms (Cerenius et al., 2008; Hancock and Sahl, 2006; Imler and Bulet, 2005; Kanost et al., 2004; Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007; Marmaras and Lampropoulou, 2009). ProPO activation can be triggered by bacterial components after their recognition by specific PRRs, leading to activation of a serine proteinase cascade that results in activation of proPO-activating proteinases (PAPs) (Cerenius et al., 2008). Active PAPs then convert proPO to functionally active phenoloxidase (PO) in the presence of essential co-factors, serine proteinase homologs (SPHs) (Jiang et al., 2003; Yu et al., 2003), and PO is the key enzyme in the melanization reaction. For example, LPS and PG can stimulate proPO activation after binding to C-type lectins and PGRPs, respectively (Takehana et al., 2002; Yu et al., 1999; Yu and Kanost, 2000). LPS from Gram-negative bacteria, LTA from Gram-positive bacteria, and PG from both Gram-negative and Gram-positive bacteria are three major bacterial components. However, it is not clear which bacterial component (LPS, LTA or PG) is more potent in activation of proPO.

Expression of AMPs is regulated by signal transduction pathways. In *Drosophila melanogaster*, AMP expression is regulated by the Toll and immune deficiency (IMD) pathways (Lemaitre and Hoffmann, 2007), and the two pathways are mainly activated by Lys (Lysine)-type and DAP (Diaminopimelic acid)-type PGs, respectively (Choe et al., 2005; Michel et al., 2001). Most Gram-positive bacteria contain the Lys-type PG, while Gram-negative bacteria and Gram-positive *Bacilli* species contain the DAP-type PG (Schleifer and Kandler, 1972). Interestingly, the DAP in *Bacilli* species is mostly amidated-DAP (more than 90%) but the DAP in Gram-negative bacteria is a meso-DAP (Leulier et al., 2003; Schleifer and Kandler, 1972). LPS and LTA are also potent elicitors in activation of immune-related genes in several insect species, including *Tribolium castaneum* (Altincicek et al., 2008), *D. melanogaster* (Jin et al., 2008), *Tenebrio molitor* (Haine et al., 2008), *Bombus ignitus* (Choi et al., 2008), *Bombyx mori* (Ha Lee et al., 2007), and *Thermobia domestica* (Altincicek and Vilcinskas, 2007). Although it was reported that crude LPS can induce immune responses in *D. melanogaster* (Imler et al., 2000), highly purified LPS does not activate the Toll or IMD pathway in adult flies (Kaneko et al., 2004; Leulier et al., 2003). Crude LPS are often contaminated with trace amounts of PG, which may cause activation of immune responses (Ha Lee et al., 2007; Imler et al., 2000). Recently, it was reported that highly purified LPS (TLRgrade™) can activate immune responses in the fat body of *B. mori* (a lepidopteran insect), but the activation level is lower than that by crude LPS or PG (Tanaka et al., 2009). Thus, it is necessary to confirm activation of AMPs by LPS in another lepidopteran insect. Insects encode a variety of AMPs. Some AMPs, such as Cecropin and Attacin, are commonly found in insect species, but some other AMPs, like Moricin, Lebocin and Gloverin, have been identified only in lepidopteran species but not in dipteran species (Axen et al., 1997; Cheng et al., 2006; Huang et al., 2009; Yamakawa and

Tanaka, 1999). Little is known about how these lepidoptera-specific AMPs are activated and regulated.

Smooth LPS is composed of three moieties: O-specific antigen (O-polysaccharide), carbohydrate core, and lipid A (Raetz and Whitfield, 2002). It has been demonstrated in mammals that lipid A moiety of LPS can stimulate the TLR4 signaling pathway (Meng et al., 2010). However, it is not known whether the O-specific antigen and core moieties of LPS play a role in the TLR4 signaling pathway or not. Recently, LTA has been identified as a ligand for Draper in phagocytosis of *Staphylococcus aureus* by *Drosophila* hemocytes (Hashimoto et al., 2009). However, no study thus far has been reported about activation of AMPs by LTA in model insects like *D. melanogaster* and *B. mori* or in other insects. Here we report AMP expression activated by smooth LPS (TLR4grade), LTA and PG from the respective bacterial strains, and by synthetic lipid A and rough mutants (Ra and Re) of LPS (TLRgrade™), in the hemocytes and fat body of larvae from the tobacco hornworm, *M. sexta*. We also compared the ability of LPS, LTA and PG in proPO activation using *M. sexta* larval plasma. Our results showed that DAP-type PGs (from *Escherichia coli* and *Bacillus subtilis*) are major elicitors of proPO activation in *M. sexta* larval plasma compared to LPS and LTA from the respective bacterial strains and a Lys-type PG from *S. aureus*. Smooth LPS, two rough mutants (Ra and Re) of LPS, synthetic lipid A, LTA and PG all significantly induced expression of AMP genes to various levels in the hemocytes and fat body of *M. sexta* larvae compared to the controls (injection with saline or DMSO). However, the activation levels of AMPs by LPS-K12 (from *E. coli* K12) and LTA-BS (from *B. subtilis*) were significantly higher than those by PG-K12 and PG-BS from the respective bacterial strains (equal amounts of bacterial components), respectively, suggesting that LPS-K12 and LTA-BS can indeed activate AMP expression in *M. sexta* larvae. But PG-SA (from *S. aureus*) was more potent than LTA-SA in activation of AMP expression. We also observed that lipid A and two rough mutants (Ra and Re) of LPS activated expression of AMP genes to significantly lower levels than smooth LPS did. Our results demonstrated for the first time that LTA can activate AMP expression, and different moieties of LPS may have synergistic effects on activation of AMPs in *M. sexta*.

## 2. Material and methods

### 2.1. Insects and bacterial cell wall components

*M. sexta* eggs were kindly provided by Professor Michael Kanost, Department of Biochemistry at Kansas State University. Larvae were reared on artificial diet at 25°C (Dunn and Drake, 1983), and 5$^{th}$ instar larvae were used for all experiments. Ultrapure LPS (TLR4grade, only activates the TLR4 pathway) from *E. coli* strain 0111:B4 (LPS-0111) and strain K12 (LPS-K12) and PG from the same *E. coli* strains (PG-0111 and PG-K12), LTA and PG from *Staphylococcus aureus* (LTA-SA, PG-SA) and *Bacillus subtilis* (LTA-BS, PG-BS) were purchased from InvivoGen (San Diego, California, USA). Synthetic lipid A was purchased from Peptide Institute, Inc (Minoh-shi, Osaka, JAPAN). Rough mutants of LPS from *E. coli* EH100 (LPS-Ra, TLRgrade™) and *E. coli* R515 (LPS-Re, TLR4grade™) were purchased from Alexis Biochemicals (Plymouth Meeting, PA, USA).

### 2.2 *In vitro* proPO activation

Individual *M. sexta* larval hemolymph samples were collected, hemocytes were removed by centrifugation and plasma samples were screened for proPO activation as described previously (Jiang et al., 2001). Plasma samples with low PO activity when incubated at room temperature alone but high PO activity when incubated with *Micrococcus luteus* were used for proPO activation assays. Plasma sample #15 was selected and used for the following experiments. Aliquots (2 μL each) of plasma were incubated alone or with 1 μg of

LPS-K12, PG-K12, LTA-BS, PG-BS, LTA-SA or PG-SA in a total of 10 μL Tris-$Ca^{2+}$ buffer (100 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, pH 7.5) in wells of a 96-well microtiter plate for 30 min at room temperature, then L-dopamine substrate solution (2 mM L-dopamine in 50 mM phosphate buffer, pH 6.5) (190 μL) was added. Absorbance at 470nm was measured every 30 seconds for a total of 30 readings with a microtiter plate reader (PowerWave XS, Bio-Tek Instrument, Inc). One unit of PO activity is defined as an increase of absorbance ($\Delta A_{470}$) by 0.001 per minute. Data from four replicates of each sample were analyzed. Experiments were repeated 3 times and similar results were obtained. To determine whether LPS-K12 and LTA-BS can activate proPO after prolonged incubation, aliquots of plasma were incubated with LPS-K12 and LTA-BS for a total of two hours. PO activity was measured every 30 min and data were collected as described above.

### 2.3 Bacterial inhibition zone assay

The inhibition zone assay was performed according to a published protocol (Hultmark, 1998). A single colony of *E. coli* DH5α or *B. subtilis* was grown overnight in Luria-Bertani (LB) broth at 37°C. Overnight bacterial cultures (0.1mL) were then inoculated into fresh LB broth (5 mL) and bacteria were grown to late log-phase. Log-phase bacterial cultures were diluted to $2 \times 10^8$ cfu/mL, and 1μL bacterial culture (about 200,000 cfu) was added into 5 mL LB containing 0.8% agarose (kept in a 45°C heating block), mixed well and spreaded immediately on a petri dish. After the medium was solidified, 2-mm wells were punched in the agarose, four wells per plate. Hemolymph was collected from naïve larvae or larvae injected with bacterial components (10 μg per larva) at 24 h post-injection (at least four larvae per group), and hemocytes were removed by centrifugation. Plasma samples were collected (from at least 4 larvae) and 4 μL plasma samples were added into each well. The plates were incubated at 37°C overnight, and the diameters of inhibition zones were measured (by subtracting 2-mm from the punched well) and recorded.

### 2.4 *In vivo* immune stimulation in *M. sexta* larvae by bacterial components

Fifth instar *M. sexta* larvae were anesthetized on ice for 20 min. Each larva was injected with saline (control) or with 20 μg of Lipid A, LPS-Ra, LPS-Re, LPS-0111, LPS-K12, PG-0111, PG-K12, PG-SA, PG-BS, LTA-SA or LTA-BS (four larvae per group). Hemolymph was collected 24 h post-injection and mixed with equal volume of anti-coagulant (AC) saline (4 mM NaCl, 40 mM KCl, 8 mM EDTA, 9.5 mM citric acid-monohydrate, 27 mM sodium citrate, 5% sucrose, 0.1% polyvinylpyrollidone, 1.7 mM PIPES) supplemented with propylthiouracil. Hemocytes were collected after centrifugation at 3000g for 10 min at 4°C and immediately resuspended in 1 mL TRI reagent (Sigma Aldrich) for total RNA extraction. Fat body was also collected from these larvae and washed three times in AC saline for total RNA extraction. For reverse transcription, total RNA (2 μg) was treated with RQ1 RNase-free DNase I (Promega) at 37°C for 30 min to remove contaminated genomic DNA, and DNase I was inactivated by heating to 75°C for 20 min. Reverse transcription was performed using OligodT primer (Promega) and ImProm-II reverse transcriptase (Promega) following the manufacturer's instructions. RNase H (NEB) was added to remove hybrid RNA, and cDNA was diluted 10-fold with distilled water. Real-time PCR was performed with SYBR Premix (Takara) on a 7500 system (Applied Biosystems) using diluted cDNAs as templates and primers listed in Table 1. Data from three replicates of each sample were analyzed with SDS software (ABI) using a comparative method ($2^{-\Delta\Delta Ct}$). Samples from saline-injected larvae were used as the controls.

For stimulation by different forms of LPS, larvae were injected with DMSO, lipid A (dissolved in DMSO), LPS-Ra, LPS-Re, LPS-0111 and LPS-K12 (20 μg per larva) (four larvae per group), hemocytes were collected at 24h post-injection for real-time PCR analysis as described above. All the above experiments were repeated from three independently

pooled samples (biological repeats, n=3), similar results were obtained, and figures in this article represent a typical set of data.

### 2.5 *In vivo* dose-dependent immune stimulation in *M. sexta* larvae by LPS, PG, and LTA

For dose-dependent stimulation assay, each larva was injected with 0.02, 0.2 or 2 μg of LPS-K12, PG-K12, LTA-BS, PG-BS, LTA-SA, or PG-SA (four larvae per group). Hemocytes were collected at 6h post-injection for preparation of RNAs and analysis of AMP gene expression in these hemocytes was performed by real-time PCR the same as described above. These experiments were repeated three times.

### 2.6 *In vitro* immune stimulation in the hemocytes and fat body by bacterial components

Hemolymph from six $5^{th}$ instar *M. sexta* naïve larvae was collected into an equal volume of AC saline, and hemocytes ($5\times10^6$) were seeded in wells of 6-well plates. After adhesion, hemocytes were washed three times with the Grace's medium containing 1× penicillin and streptomycin (HyClone) to remove plasma, then 2 mL of the Grace's medium were added into each well. Larval fat body was also collected from naïve larvae (at least 4 larvae) into AC saline, and dispensed into wells of 6-well plates. Fat body was washed three times with the Grace's medium, and 2 mL of the Grace's medium were added to each well. LPS-K12, LTA-BS, PG-K12 or PG-BS (20 μg per well) was added to each well, and hemocytes and fat body were incubated for 24 h at 25°C. Saline (20 μL of 0.85% NaCl) was added in the control wells. Total RNA was prepared from these hemocytes and fat body, and gene expression was analyzed by real-time PCR as described above. These experiments were repeated three times.

### 2.7 Data analysis

Figures were made with GraphPad Prism software with one representative set of data. The significance of difference was determined by an unpaired t-test or by ANOVA followed by a Tukey's multiple comparison test with the same software (GraphPad, San Diego, CA).

## 3. Results

### 3.1 Prophenoloxidase is mainly activated by DAP-type PGs

Activation of prophenoloxidase (proPO) is an important immune mechanism in arthropods against large parasites, and the proPO activation cascade can be triggered by bacterial components when specific recognition proteins are present (Takehana et al., 2002; Yu et al., 1999; Yu and Kanost, 2000). We used an *in vitro* proPO activation assay to test which bacterial components are able to activate proPO in *M. sexta* larval plasma. When the naïve plasma samples were incubated at room temperature for 30 min, very low PO activity was observed in the plasma sample alone and in the plasma samples incubated with LPS-K12 (from *E. coli* K12), LTA-BS (from *B. subtilis*) and LTA-SA (from *S. aureus*), indicating that proPO was not activated by LPS or LTA under these conditions (Fig. 1A). However, significantly higher PO activity was detected in the naïve plasma samples incubated with three PGs (PG-K12, PG-BS and PG-SA) compared to the plasma alone, and the two DAP-type PGs (PG-K12 and PG-BS) activated PO activity (~60 units) to a significantly higher level than the Lys-type PG (PG-SA) did (~6 units) (Fig. 1A).

It was reported that synthetic lipid A at high concentrations can activate proPO in the silkworm larval plasma (Kaneko et al., 2004). To further determine whether LPS-K12 and LTA-BS can activate proPO in *M. sexta* plasma, naïve plasma samples were incubated with LPS-K12 and LTA-BS for a longer period of time (a total of 2 h). Compared to the naïve plasma alone, addition of LPS-K12 and LTA-BS increased PO activity in the plasma samples after prolonged incubation (except for LTA-BS at 60 min) (Fig. 1B). But the

amplitude of PO activity (1.2–3.0 units) activated by LPS and LTA after prolonged incubation was lower than that (~ 6 units) activated by the Lys-type PG-SA, and was significantly lower than the PO activity (~60 units) activated by the DAP-type PG-K12 and PG-BS (Fig. 1B). These results suggest that *M. sexta* proPO is activated rapidly and strongly by DAP-type PG-K12 and PG-BS. LPS-K12 and LTA-BS may activate proPO with prolonged activation time, but they are much less potent than PGs in activation of proPO.

### 3.2 Antibacterial activity in *M. sexta* larvae is activated by bacterial components

To determine whether LPS, LTA and PG can activate AMP expression in *M. sexta* larvae, antibacterial activity of plasma from *M. sexta* naïve larvae injected with bacterial components was measured by a bacterial inhibition zone assay. Plasma from saline-injected larvae did not show antibacterial activity against either Gram-negative *E. coli* or Gram-positive *B. subtilis* since no inhibition zones were formed (Fig. 2A). However, plasma samples from larvae injected with LPS-K12, LTA-BS and PG-SA showed high antibacterial activity against both *E. coli* and *B. subtilis*, while plasma samples from PG-K12 and PG-BS (DAP-type PGs) as well as LTA-SA injected larvae showed antibacterial activity only to *E. coli* but not to *B. subtilis*.

Lysozyme has been identified in *M. sexta* (Mulnix and Dunn, 1994), and it is active against Gram-positive bacteria. To test whether increase in the activity against Gram-positive *B. subtilis* in the plasma is due to activation of lysozyme in *M. sexta* larvae by bacterial components, a real-time PCR was performed. Three PGs (PG-SA, PG-BS and PG-K12) activated lysozyme expression in the hemocytes to a similar low level, LPS-K12 increased lysozyme expression to a significantly higher level than the three PGs did ($p<0.05$), LTA-BS activated lysozyme expression to the highest level among the six bacterial components tested, and LTA-SA almost did not activate lysozyme expression (Fig. 2B). However, the plasma samples of larvae injected with PG-SA and LTA-BS showed similar high activity against *B. subtilis*, which is signigicantly higher than the activity in the LPS-K12 activated plasma ($p<0.05$), and no activity against *B. subtilis* was detected in the plasma samples activated by PG-BS, PG-K12 and LTA-SA (Fig. 2A). These results suggest that increase in lysozyme expression may contribute very little to the activity against *B. subtilis* in the plasma.

### 3.3 Bacterial components activate antimicrobial peptide genes in *M. sexta* larvae

In insects, antimicrobial peptides (AMPs) are small peptides synthesized and secreted mainly by fat body and hemocytes. Expression of AMPs in *D. melanogaster* is induced primarily by PGs and regulated by the Toll and IMD pathways (Lemaitre and Hoffmann, 2007), but LPS cannot activate immune responses in adult flies (Kaneko et al., 2004; Leulier et al., 2003). Highly purified (TLRgrade™) LPS can activate immune responses in the fat body of *B. mori* to a lower level than that by crude LPS or PG (Tanaka et al., 2009). So far, it is not known whether LTA can activate AMP expression in insects. Thus, we first tested induction of AMP genes in hemocytes and fat body of *M. sexta* larvae by LPS, LTA and PG. The real-time PCR results showed that expression of Attacin, Lebocin and Moricin in both hemocytes and fat body of *M. sexta* larvae was significantly induced by all the bacterial components tested at 24h post-injection, including smooth LPS, LTA and both DAP-type and Lys-type PGs, although the activation levels varied with different bacterial components (Fig. 3). It is noteworthy that for LTA/PG from Gram-positive *B. subtilis*, LTA-BS activated the AMP genes to a significantly higher level than PG-BS did, but for LTA/PG from Gram-positive *S. aureus*, the activation levels of AMP genes were significantly lower by LTA-SA than by PG-SA (Fig. 3). For LPS/PG from *E. coli* strain K-12, LPS-K12 (TLR4grade) activated AMP genes expression to a comparable high level as PG-K12 or to a higher level than PG-K12 did (Fig. 3).

### 3.4 LPS-K12 and LTA-BS are potent elicitors in activation of antimicrobial peptide genes

To confirm that LPS-K12 and LTA-BS can indeed activate expression of AMP genes in *M. sexta* larvae, a dose-dependent activation assay was performed using less but equal amounts of LPS-K12, PG-K12, LTA-BS, PG-BS, LTA-SA and PG-SA, and short activation time (6h post-injection) (Fig. 4). Real-time PCR results showed that expression of Attacin, Lebocin and Moricin genes in the hemocytes at 6h post-injection was significantly higher in the larvae injected with 0.02 μg of LPS-K12, LTA-BS, PG-BS, LTA-SA and PG-SA than in the control larvae (injected with saline) (Fig. 4), but the same amount (0.02 μg) of PG-K12 almost did not activate expression of Attacin and Lebocin (Fig. 4A and B) compared to the control groups. The activation levels of AMP genes were significantly higher in the larvae injected with increasing amounts of LPS, LTA and PG (Fig. 4), indicating a dose-dependent activation of AMP genes by these bacterial components. More importantly, LPS-K12 and LTA-BS activated expressions of AMP genes to significantly higher levels than PG-K12 and PG-BS did, respectively, at all three doses (Fig. 4A–F), but LTA-SA activated AMP expression to a significantly lower level than PG-SA did at all three concentrations (Fig. 4G–I). These results suggest that LPS-K12 and LTA-BS indeed can stimulate AMP gene expression in *M. sexta* larvae.

### 3.5 Lipid A, core carbohydrate and O-specific antigen moieties of LPS may synergistically activate AMP expression

Smooth LPS is composed of the O-polysaccharide chain (O-antigen), the core (outer and inner core) polysaccharide, and lipid A moieties (Raetz and Whitfield, 2002). Each moiety of LPS may induce immune responses differently (Huber et al., 2006). We compared the induction of AMP genes in *M. sexta* larvae by lipid A, two rough mutants (Ra and Re) of LPS and two smooth LPS from two *E. coli* strains. LPS-Re is comprised of lipid A and 2-keto-3-deoxyoctonate (KDO), while LPS-Ra lacks only the O-antigen moiety. Injection of lipid A increased expressions of Attacin, Lebocin and Moricin in hemocytes to a low but significantly higher level compared to the DMSO (solvent for Lipid A) control group, and LPS-Re also activated expression of Attacin and Lebocin to a low but significantly higher level than the saline-injection group (Fig. 5). LPS-Ra and two smooth LPS (LPS-0111 and LPS-K12) all activated expression of the three AMP genes in the hemocytes to significantly higher levels than LPS-Re and lipid A did (Fig. 5). The activation level of AMP genes by LPS-K12 and LPS-0111 (smooth LPS) was significantly higher than that by LPS-Ra (Fig. 5). Together, these results suggest that lipid A, the core polysaccharide, and the O-antigen moieties may all induce AMP expression, and the O-antigen moiety may be more important in stimulating immune responses in *M. sexta*.

### 3.6 Bacterial components activate antimicrobial peptide genes in fat body and hemocytes *in vitro*

Highly purified LPS can activate AMP expression in the fat body of *B. mori*, but does not activate AMP genes in a cell line derived from the *B. mori* fat body (Tanaka et al., 2009), suggesting that some plasma factors may play an important role in LPS recognition. To test whether plasma factors/proteins are required for stimulation of AMP expression by bacterial components in *M. sexta*, hemocytes and fat body were collected from naïve larvae separately and then cultured *in vitro* in the presence of LPS-K12, PG-K12, LTA-BS and PG-BS. Expression of AMP genes was determined by real-time PCR. We found that all four bacterial components could induce expression of Attacin, Lebocin and Moricin in both hemocytes (Fig. 6A–C) (except PG-K12 for Attacin in Fig. 6A) and fat body (Fig. 6D–F) *in vitro* to various low levels, but *in vitro* activation of AMPs (Fig. 6) was several orders lower than *in vivo* activation (Fig. 3).

## 4. Discussion

Insects have effective innate immune responses, including activation of prophenoloxidase (proPO) and synthesis of AMPs. The proPO activation system is a complicated process that involves a cascade of serine proteinases and other hemolymph proteins (Cerenius et al., 2008; Jiang et al., 2003; Yu et al., 2003). ProPO activation can be triggered by different microbial components after their binding to specific recognition proteins. We compared the ability of LPS, LTA and PG in activation of proPO in *M. sexta* plasma. Surprisingly, PG-K12 (from *E. coli* K12) and PG-BS (from *B. subtilis*), two DAP-type PGs which were less potent than LPS-K12 and LTA-BS, respectively, in activation of AMP expression (Figs. 3 and 4), were more potent elicitors in proPO activation (Fig. 1A). PG-SA (from *S. aureus*), a Lys-type PG, also activated PO (~6 units), but to a significantly lower level than PG-K12 and PG-BS did (~60 units) (Fig. 1B). LPS-K12 and LTA-BS activated PO to a low level even after prolonged incubation (Fig. 1B). These results suggest that PG is a stronger elicitor in proPO activation compared to LPS and LTA from the respective bacterial strains, and DAP-type PG is more potent than Lys-type PG in proPO activation.

In *D. melanogaster*, bacteria-activated expression of AMPs is mainly induced by PGs and is regulated by the Toll and IMD pathways (Kaneko et al., 2004; Michel et al., 2001). However, highly purified LPS, a major component of Gram-negative bacteria, cannot activate the Toll or IMD pathway in adult flies (Kaneko et al., 2004; Leulier et al., 2003), although LPS weakly activates AMP production in the fat body of *B. mori* (Tanaka et al., 2009). In addition, there has been no report about the activation of AMPs in insects by LTA, another major component of Gram-positive bacteria. In this study, we used smooth and rough mutants of LPS (TLR4grade, TLR4grade™ and TLRgrade™), LTA and PG to activate AMP expression in the tobacco hornworm, *M. sexta*. Our results showed that LPS, LTA and PG all were able to stimulate expression of AMPs in hemocytes and fat body of *M. sexta* larvae (Figs. 3–5), and activation of AMPs by these bacterial components was dose-dependent (Fig. 4).

LPS and LTA preparations may be contaminated with small amounts of PGs, which are potent elicitors in activation of the Toll and IMD pathways in *Drosophila* (Kaneko et al., 2004; Leulier et al., 2003). The bacterial components used in this study are high quality preparations. LPS-K12 and LPS-0111 (smooth LPS) are TLR4grade (only activates TLR4), and LPS-Ra and LPS-Re (rough mutants of LPS) are TLRgrade™ and TLR4grade™ that have been tested in mammalian cells. The LPS used in activation of immune responses in the fat body of *B. mori* [36] was TLRgrade™ from the same company (Alexis Biochemicals). LTA-BS and LTA-SA are also high quality samples with only 12.5 and 125 EU (endotoxin units)/mg of LTA-BS and LTA-SA, respectively. The endotoxin contamination in the three PGs was also very low (endotoxin-free for PG-SA, 0.25 and 125 EU/mg for PG-BS and PG-K12, respectively). Even so, we still cannot rule out the trace amounts of PGs in LPS and LTA samples. In order to confirm that activation of AMPs by LPS and LTA is indeed by the respective bacterial components but not by any contaminated PGs, activation levels of AMPs by LPS and LTA were compared with those by PGs from the respective bacterial strains at three different doses (Fig. 4). We clearly showed that LPS-K12 (from *E. coli* K-12) and LTA-BS (from *B. subtilis*) were more potent than PG-K12 and PG-BS, respectively, in activation of AMP expression in hemocytes at three different doses tested (Fig. 4A–F), indicating that LPS-K12 and LTA-BS can activate AMP expressions in *M. sexta* larvae. To our knowledge, this is the first report about activation of AMPs in insects by LTA-BS. But we also observed that LTA-SA (from *S. aureus*) activated AMP expression to a significantly lower level than PG-SA did (Fig. 4G–I). Interestingly, LTA-SA is the least potent elicitor among the six bacterial components tested in activation of the

AMP genes and lysozyme in *M. sexta* (Figs. 2B and 3, except for Attacin activation in fat body, Fig. 3A).

It has been demonstrated that LPS cannot activate the IMD or Toll pathway in *D. melanogaster* (Kaneko et al., 2004; Leulier et al., 2003). Our results clearly showed that LPS-K12 could activate AMP expression in *M. sexta* larvae (Figs. 3–5), a result consistent with that of *B. mori* (Tanaka et al., 2009). Since *B. mori* and *M. sexta* are lepidopteran insects, while *D. melanogaster* is a dipteran insect, one possibility is that lepidopteran and dipteran insects may use different PRRs for recognition of bacterial components. For example, in *D. melanogaster*, there are 13 PGRP genes encoding about 20 functional PGRP proteins (Charroux et al., 2009) that can serve as PRRs to recognize PGs from both Gram-negative and Gram-positive bacteria (Royet, 2004; Royet and Dziarski, 2007). In *M. sexta* and *B. mori*, C-type lectins and other plasma proteins that can bind LPS and LTA may function as PRRs for recognition of LPS and LTA (Lee et al., 1996; Ohta et al., 2006; Watanabe et al., 2006; Xu et al., 1995; Yu et al., 2005; Yu and Kanost, 2000; Yu et al., 2006).

Smooth LPS is composed of the O-antigen, outer core, inner core, and lipid A moieties, and rough mutants of LPS (R form LPS) lack the O-antigen moiety and parts of the outer and/or inner cores (Huber et al., 2006). It is not clear whether different moieties of LPS can activate AMP expression. We showed that two smooth LPS (LPS-K12 and LPS-0111), two rough mutants of LPS (LPS-Re and LPS-Ra) and lipid A could all activated AMP expression in hemocytes of *M. sexta* larvae, however, smooth LPS (LPS-K12 and LPS-0111) stimulated AMP expression to a significantly higher level than rough LPS (LPS-Re and LPS-Ra) and lipid A did (Fig. 5). These results suggest that multiple moieties of LPS may be recognized by different PRRs simultaneously, leading to a synergistic effect in stimulation of AMP expression in insects, a phenomenon that has not been reported in mammals in which lipid A is the major moiety in stimulating immune reactions (Meng et al., 2010; Raetz and Whitfield, 2002). It is also possible that the O-antigen moiety of LPS may interfere with binding of the core moiety and/or lipid A moiety to their receptors if the receptor for the O-antigen is not present at the same time. Thus, not all smooth LPS can activate AMP expression to a higher level than rough mutants of LPS.

Activation of AMPs in *in vitro* cell culture of *M. sexta* larval hemocytes and fat body in the presence of LPS, LTA and PG was also observed (Fig. 6). However, *in vitro* activation level was several orders lower compared to *in vivo* activation (Figs. 3 and 4). These results suggest that some plasma factors/proteins may be required for recognition of LPS, LTA and PG. These plasma factors/proteins may facilitate binding of bacterial components to receptors or serve as co-receptors in the receptor complexes to activate AMP genes. In the absence of plasma factors/proteins in the *in vitro* cell culture, very little bacterial components bound to hemocytes and fat body, resulting in low activation level of AMPs. Plasma proteins with such functions may include C-type lectins, PGRPs and beta-glucan recognition/Gram negative bacteria binding proteins, which have the ability to bind LPS, LTA and PG (Ao et al., 2008a;Jomori and Natori, 1991;Lee et al., 2000;Metheniti et al., 2003;Xu et al., 1995;Yu and Kanost, 2002;Yu et al., 2006).

In *M. sexta*, five AMP genes (Attacin, Cecropin, Moricin, Lebocin and Gloverin) and a lysozyme have been identified (Gorman et al., 2004; Mulnix and Dunn, 1994). The five AMPs have activity mainly against Gram-negative bacteria, while lysozyme is active against Gram-positive bacteria. We measured antibacterial activity in the plasma of larvae injected with bacterial components against Gram-negative *E. coli* and Gram-positive *B. subtilis*. High activity against *E. coli* was detected in the plasma samples of the larvae activated by all six bacterial components tested, but activity against *B. subtilis* was detected only in the

larvae activated by LPS-K12, LTA-BS and PG-SA but not in the larvae injected with PG-K12, PG-BS and LTA-SA (Fig. 2A). Real-time PCR showed that the three PGs (PG-SA, PG-BS and PG-K12) activated lysozyme expression to a similarly low level, LPS-K12 and LTA-BS activated lysozyme expression to significantly higher levels than the three PGs did, and LTA-SA did not activate lysozyme expression (Fig. 2B). However, the activity against *B. subtilis* was significantly higher in the larvae activated by PG-SA than by LPS-K12, and no activity against *B. subtilis* was detected in the larvae activated by PG-K12, PG-BS and LTA-SA. These results indicate that activation of lysozyme has trivial effect on the activity against *B. subtilis* in the plasma. PG-SA is a Lys-type PG that can activate the Toll pathway, while PG-K12 and PG-BS are DAP-type PGs, which can activate the IMD pathway. It is likely that some yet unidentified *M. sexta* AMPs with activity against Gram-positive bacteria are activated through the Toll pathway, resulting in the activity against *B. subtilis*. One such candidate AMP gene may be defensin, as defensin is active against Gram-positive bacteria, and it has been identified in *B. mori* (Kaneko et al., 2008). Thus, activity against *E. coli* in *M. sexta* larvae activated by the six bacterial components may result from activation of AMPs via the Toll and/or IMD pathways, but the activity against *B. subtilis* in the larvae activated by PG-SA, LPS-K12 and LTA-BS may be due to activation of the Toll pathway. A Toll receptor has been identified in hemocytes of *M. sexta* larvae (Ao et al., 2008b). This *M. sexta* Toll receptor could be up-regulated to a significantly higher level by PG-SA, LPS-K12 and LTA-BS than by LTA-SA, PG-K12 and PG-BS, respectively (data not shown), a result consistent with that of the antibacterial activity assay (Fig. 2A), further supporting the hypothesis that PG-SA, LPS-K12 and LTA-BS may activate the Toll pathway, while PG-K12, PG-BS and LTA-SA may activate the IMD pathway. The difference in activation of AMP genes and signaling pathways by LTA-BS and LTA-SA may lie in different structures of LTA and teichoic acid in *B. subtilis* and *S. aureus* (Greenberg et al., 1996; Morath et al., 2002), as well as different recognition proteins/receptors in insects for LTA-BS and LTA-SA. Future work is to investigate how signaling pathways are activated by LPS and LTA in *M. sexta*.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| AC | anti-coagulant |
| AMP | antimicrobial peptide |
| IMD | immune deficiency |
| LPS | lipopolysaccharide |
| LTA | lipoteichoic acid |
| PRR | pattern recognition receptor |
| PAMP | pathogen-associated molecular pattern |
| PG | peptidoglycan |
| PGRP | peptidoglycan recognition protein |
| PO | phenoloxidase |
| proPO | prophenoloxidase |
| TLR | Toll-like receptor |

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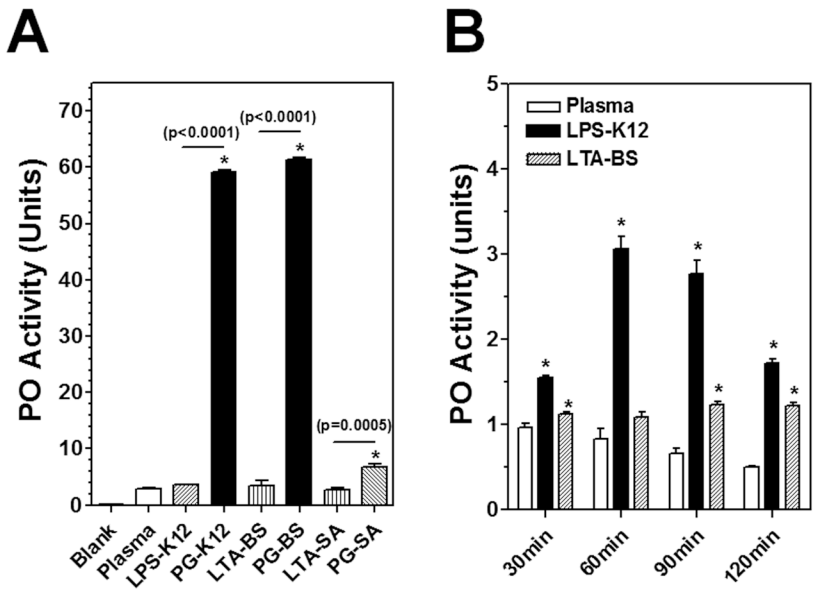


**Fig. 1. *In vitro* proPO activation in *M. sexta* larval plasma by LPS, LTA and PG**

(A) Aliquots of naïve plasma were incubated alone, or with LPS-K12, PG-K12, LTA-BS, PG-BS, LTA-SA or PG-SA in wells of a 96-well microtiter plate for 30 min at room temperature, and PO activity was determined as described in the Materials and Methods. Data from four replicates of each sample were analyzed. (B) Aliquots of naïve plasma were incubated at room temperature alone, or with LPS-K12 or LTA-BS for 30, 60, 90 or 120 min, and PO activity was determined. Each assay was performed in four replicates. The bars represent the mean of four individual measurements ± S.E.M. The significance of difference was determined by an unpaired t-test using the GraphPad InStat software. Asterisks (*) indicate significant difference compared to the naïve plasma alone group. Significance between two groups was indicated by a horizontal line and *p* value was shown above the line.

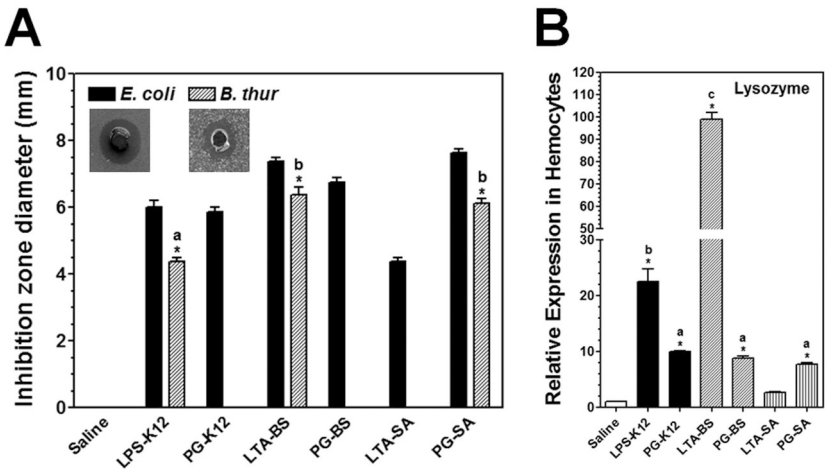


**Fig. 2. Induced antibacterial activity in the plasma and induction of lysozyme in *M. sexta* larvae by bacterial components**

*M. sexta* 5th instar naïve larvae were injected with LPS-K12, PG-K12, LTA-BS, PG-BS, LTA-SA, PG-SA or with saline (control), and hemolymph was collected at 24h post-injection. Plasma (cell-free hemolymph) samples were collected for determination of antibacterial activity by an inhibition zone assay (A), while hemocytes were collected for total RNA preparation for real-time PCR analysis of lysozyme expression (B). The bars represent the mean of four (A) or three (B) individual measurements ± S.E.M. The inset in (A) showed typical inhibition zones against *E. coli* and *B. subtilis*. Identical letters are not significant difference between groups ($p>0.05$), while different letters indicate significant difference between groups ($p<0.05$) determined by ANOVA followed by a Tukey's multiple comparison test.

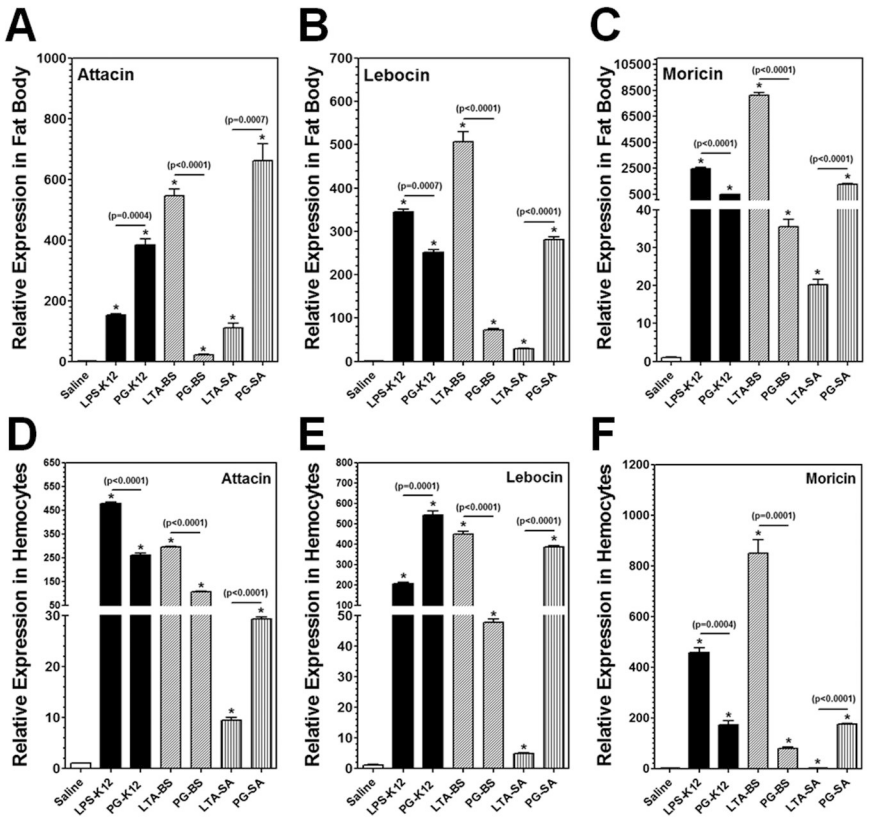


**Fig. 3. *In vivo* activation of AMPs in hemocytes and fat body of *M. sexta* larvae by LPS, LTA and PG**

*M. sexta* 5th instar naïve larvae were injected with saline, or with LPS-K12, PG-K12, LTA-BS, PG-BS, LTA-SA or PG-SA (20 μg per larva), and hemocytes and fat body were collected at 24h post-injection. Expression of Attacin, Lebocin and Moricin mRNAs in the fat body (A–C) and hemocytes (D–F) was analyzed by Real-time PCR. The bars represent the mean of three individual measurements ± S.E.M. Asterisks (*) indicate significant difference compared to the saline-injection group. Significance between two groups was indicated by a horizontal line and *p* value was shown above the line.

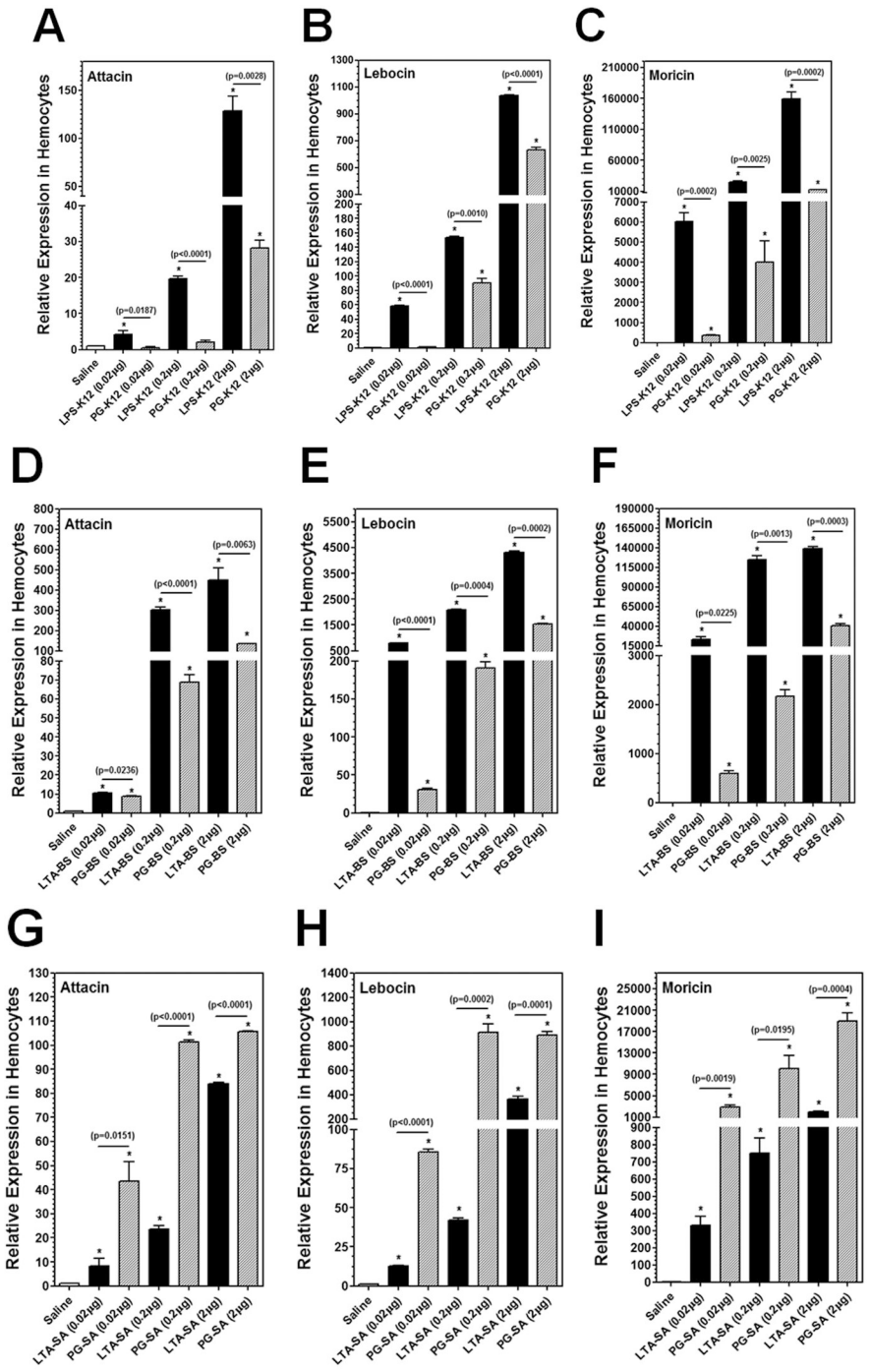


**Fig. 4. Dose-dependent activation of AMPs in hemocytes of *M. sexta* larvae by LPS, LTA and PG**

*M. sexta* 5th instar naïve larvae were injected with saline (control) or different amounts of LPS-K12 and PG-K12 (from *E. coli* K12) (A–C), LTA-BS and PG-BS (from *B. subtilis*) (D–F), or LTA-SA and PG-SA (from *S. aureus*) (G–I), and hemocytes were collected at 6h post-injection. Expression of Attacin, Lebocin and Moricin was analyzed by Real-time PCR as described in Fig. 3.

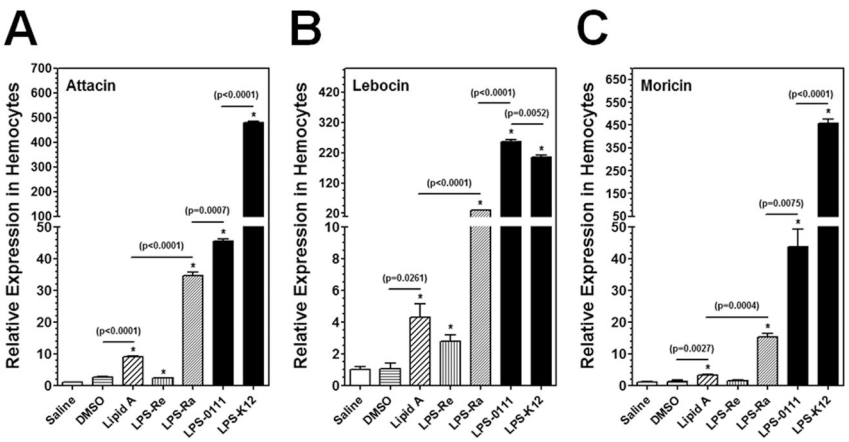


**Fig. 5. Activation of AMPs in hemocytes of *M. sexta* larvae by lipid A and different forms of LPS**

*M. sexta* 5th instar naïve larvae were injected with saline, DMSO, lipid A, LPS-Re, LPS-Ra, LPS-0111 or LPS-K12 (20 μg per larva), and hemocytes were collected at 24h post-injection. Expression of Attacin, Lebocin and Moricin was analyzed by Real-time PCR as described in Fig. 3.

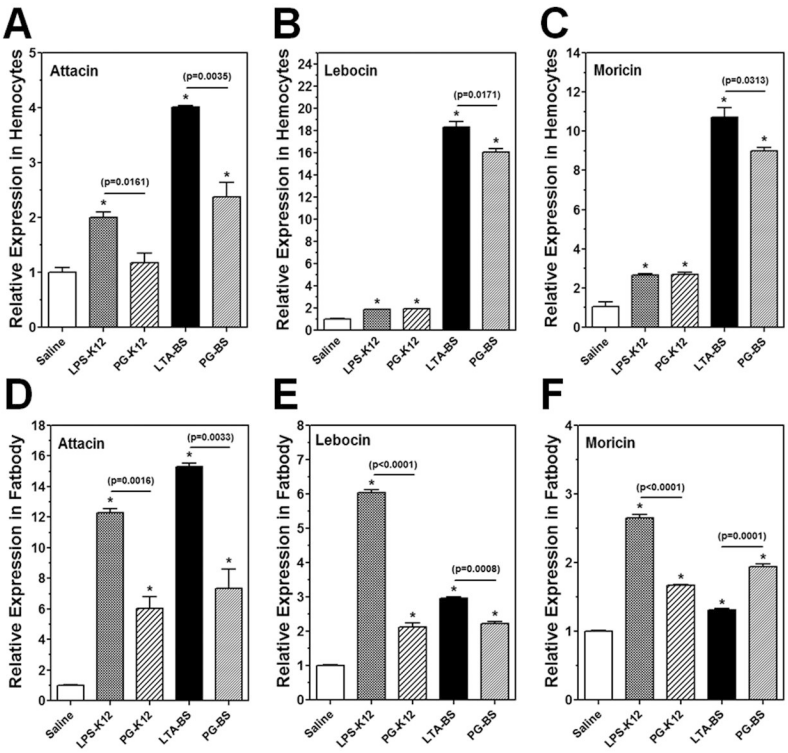


**Fig. 6. *In vitro* activation of AMPs in hemocytes and fat body of *M. sexta* larvae by LPS, LTA and PG**

Hemocytes and fat body from *M. sexta* 5$^{th}$ instar naïve larvae were collected and cultured in the Grace's medium in the presence of LPS-K12, PG-K12, LTA-BS, PG-BS or saline (control) at 25°C for 24h. Expression of Attacin, Lebocin and Moricin was analyzed by Real-time PCR as described in Fig. 3.

**Table 1**

Primers used in real-time PCR

| Gene | Forward primer | Reverse primer |
|---|---|---|
| Ribosomal protein S3 (Rps3) | 5′-GGAGCTGTACGCTGAGAAAG-3′ | 5′-TTAATTCCGAGCACTCCTTG-3′ |
| Attacin-2 | 5′-TTCGTCGTCCTGGTCTGTCT-3′ | 5′-TTGGAGAGGGAGAAGCCCAT-3′ |
| Lebocin | 5′-ACGTGCGTAGTGTGAACGAG-3′ | 5′-CGCAGATTATGAGTTACGACGA-3′ |
| Moricin | 5′-ATAAAGCAGGCTGGCAAGG-3′ | 5′-AGAAGATTCCGAAGGGAGAAC-3′ |
| Lysozyme | 5′-GTGTGCCTCGTGGAGAATG-3′ | 5′-ATGCCTTGGTGATGTCGTC-3′ |