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Interactions between *Mycoplasma pulmonis* and immune systems in the mealworm beetle, *Tenebrio molitor*

Sooa Lim[a], Hwa-Kyung Yun[b], Ki Mo Kang[c], Bok Luel Lee[d], Ran Won[e], In Hee Lee[c,*]

[a] Department of Food & Pharmaceutical Engineering, Hoseo University, Asan, Chungnam, 336-795, South Korea
[b] Department of Biofood & Medical Sciences, Hanseo University, 360 Daegok-ri, Haemi-myen, Seosan, Chungnam, 356-706, South Korea
[c] Department of Biotechnology, Hoseo University, Asan, Chungnam, 336-795, South Korea
[d] National Research Laboratory of Defense Proteins, College of Pharmacy, Pusan National University, Busan, 609-735, South Korea
[e] Department of Biomedical Laboratory Science, Division of Health Sciences, Dongseo University, Busan, 47011, South Korea

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ABSTRACT

Mycoplasmas, the smallest self-replicating organisms, are unique in that they lack cell walls but possess distinctive plasma membranes containing sterol acquired from their growth environment. Although mycoplasmas are known to be successful pathogens in a wide range of animal hosts, including humans, the molecular basis for their virulence and interaction with the host immune systems remains largely unknown. This study was conducted to elucidate the biochemical relationship between mycoplasma and the insect immune system. We investigated defense reactions of *Tenebrio molitor* that were activated in response to infection with *Mycoplasma pulmonis*. The results revealed that *T. molitor* larvae were more resistant to mycoplasma infection than normal bacteria equipped with cell walls. Intruding *M. pulmonis* cells were effectively killed by toxins generated from activation of the proPO cascade in hemolymph, but not by cellular reactions or antimicrobial peptides. It was determined that these different anti-mycoplasma effects of *T. molitor* immune components were primarily attributable to surface molecules of *M. pulmonis* such as phospholipids occurring in the outer leaflet of the membrane lipid bilayer. While phosphatidylcholine, a phospholipid derived from the growth environment, contributed to the resistance of *M. pulmonis* against antimicrobial peptides produced by *T. molitor*, phosphatidylglycerol was responsible for triggering activation of the proPO cascade.

1. Introduction

Insects have a variety of defense systems to fight against microorganisms invading their hemocoel. Over the last four decades, there has been accumulating evidence that insects have a sophisticated immune system comparable to human innate immunity (Kuo et al., 2018; Sheehan et al., 2018). Accordingly, they have served as a good model system for studying interactions between the innate immune system and microbial pathogens. When microorganisms come into the hemocoel of insects, they can be phagocytosed by hemocytes, trapped in hemocyte aggregates or nodules (Satyavathi et al., 2014; Gold and Bruckner, 2015), and killed by toxic intermediates generated from the activation of the prophenoloxidase (proPO) cascade (Eleftherianos and Revenis, 2011). Alternatively, they could succumb to a variety of antimicrobial peptides (AMPs) that appear in the hemolymph in response to infection (Tonk and Vilcinskas, 2017). It is well known that all these defense reactions begin with the recognition of invaders through a series of pattern recognition receptors including hemolymph proteins and/or surface proteins on hemocytes. Pattern recognition receptors are mainly bound to the surface molecules of microbial cell walls, referring to as pathogen-associated molecular patterns (Cerenius et al., 2008; Wang et al., 2019), such as lipopolysaccharides, peptidoglycans and β-1,3-glucan. Therefore, pathogen-associated molecular patterns of the microbial cell wall are considered to be crucial elicitors of insect immune response. If then, it would be interesting to find out how the insect defense system copes with an infection caused by microbes lacking the cell wall.

Mycoplasmas have been shown to be successful pathogens in a wide range of animal hosts, including human and etiological agents of several chronic diseases such as pneumonia, urethritis, arthritis and mastitis (Burki et al., 2015; McGowin and Totten, 2017; Kumar, 2018; Parker et al., 2018). Being the smallest and simplest self-replicating organisms, mycoplasmas exhibit unique features. For example, they have no rigid cell wall, and are surrounded by a single plasma

* Corresponding author.
E-mail address: leeih@hoseo.edu (I.H. Lee).

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Abbreviations

ProPO	prophenoloxidase
PO	phenoloxidase
AMPs	antimicrobial peptdes
PTU	phenylthiourea
CFU	colony forming unit
POPC	phosphatidylcholine
POPG	phosphatidylglycerol
GIM	Grace's insect medium
FITC	fluorescein isothiocyanate
BSA	Bovine serum albumin
Tm-SPH1	T. molitor-serine protease homolog 1

membrane that directly contacts their environment. In addition, unlike other bacteria equipped with cell walls, mycoplasmas have choline-containing phospholipids, which are incorporated into their plasma membrane from serum-containing medium. These serum-originated phospholipids are considered to be essential to mycoplasma proliferation and involved in immune evasion, as well as the virulence of mycoplasmas (Rottem, 2002; Grosshennig et al., 2013). However, comparing to ordinary bacterial pathogens, there is currently a great lack of information about the innate immune responses operating against mycoplasma infection.

This study was conducted to elucidate the interaction between mycoplasma and the insect defense systems using *M. pulmonis* and *T. molitor* in its larval stage. Since *T. molitor* (mealworm beetles) has been well studied as for their defense systems including cellular immunity (Johnston et al., 2013) proPO cascade (Zhang et al., 2004; Kan et al., 2008) and AMPs (Chae et al., 2012), it was thought to be an appropriate model insect for this study. Firstly, we evaluated the insecticidal activity of mycoplasma upon its injection into the insect hemocoel. We then investigated the resistance of mycoplasma against attacks by insect humoral and cellular defense systems. During the course of our biochemical analyses, we employed two ordinary bacteria equipped with cell walls, *Staphylococcus aureus* and *Escherichia coli*, to show the distinct action of the insect defense systems toward mycoplasma. To the best of our knowledge, this study is the first to shed light on the mechanism by which the insect immune system operates defense reactions to mycoplasma entering the hemocoel.

2. Materials and methods

2.1. Bacteria strains and cell line

Staphylococcus aureus (KCTC 1621) and *Escherichia coli* (KCCM 40880) were obtained from the Korea Culture Center of Microorganisms (KCCM, Korea) and maintained in tryptic soy broth at 37 °C. In addition, *Mycoplasma pulmonis* strain CT was kindly provided by Dr. Kevin Dybvig (Department of Genetics, University of Alabama at Birmingham) and cultured as previously described (Park et al., 2013). Briefly, *M. pulmonis* were cultured in PPLO broth supplemented with 10% heat-inactivated horse serum (GIBCO, 16050-122), glucose, ampicillin, and phenol red at 36 °C. To prepare the 1% (w/v) PPLO agar plate, the broth was mixed with the bacto agar (Difco, 214010) without phenol red. Murine RAW264.7 macrophages were obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI medium with 10% heat-inactivated FBS using 150-cm^2 T flasks while maintained at 37 °C in a 5% CO_2 environment.

2.2. Insects, immunization, and preparation of plasma or hemocytes

T. molitor larvae were maintained on a laboratory bench in terraria containing wheat bran and the last instar larvae were used for all experiments. For immunization, 2 µl of log-phase bacteria (1×10^4 CFU/larva) were injected into the hemocoels of the larvae. After being cultured for 12 h at 36 °C, hemolymph was collected into ice-cold tubes containing a few crystals of phenylthiourea (PTU). Cells and debris were then removed by centrifugation at 15,000 *g* for 10 min and 4 °C, after which the cell-free hemolymph (hereafter designated as plasma) was utilized immediately or stored at −70 °C. To harvest the hemocytes, hemolymph was collected into 1 ml of chilled anticoagulant buffer (136 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, and 15 mM sodium chloride, pH 5.0) and subsequently centrifuged at 30 *g* for 5 min at 4 °C. Finally, sediments containing hemocytes were washed twice with Grace's insect medium (GIM, Sigma). Isolated hemocytes were used immediately for experiments, although they were confirmed to be more than 90% viable at 2 h after breeding.

2.3. Infection of M. pulmonis and CFU count

T. molitor larvae were infected by injection with different doses of mycoplasma. Briefly, mycoplasma cells were washed five times by repeating centrifugation for 15 min at 2000 × *g* and then rinsing with insect saline (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, pH 6.0). Next, 2 µl of the relevant mycoplasma suspension were injected into the hemocoel of 10 larvae using a Hamilton syringe, after which the larvae were incubated at 36 °C. Insect mortality was recorded at 6 h intervals for the first 24 h, then at 24 h intervals. To examine mycoplasma survival in the insect hemocoel, larvae were injected with 2 µl of *M. pulmonis* (1×10^5 CFU/larva). Following incubation for predetermined times at 36 °C, hemolymphs were collected from three larvae in the absence of PTU. Ten microliters of hemolymph at each time point were then directly plated on PPLO agar and incubated for 10 days at 36 °C in a humid chamber, after which the viable colonies were counted. Unless we mentioned, all experiments were repeated three times and statistical

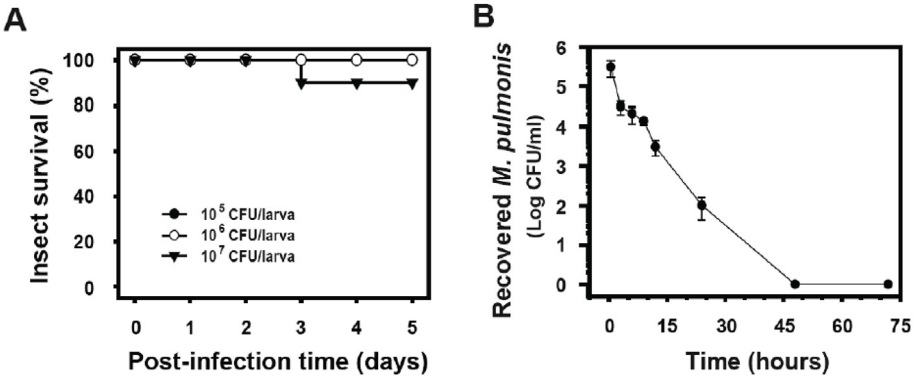


Fig. 1. Insect mortality and survival in the hemocoel of *M. pulmonis*. (A) Dose-dependent survival of *T. molitor* larvae after injection with *M. pulmonis*. Survival was monitored over 5 days post-infection. Ten larvae were used for each group. (B) *M. pulmonis*-infected larvae hemolymph was collected at predetermined times (0.5, 3, 9, 12, 24, 48, and 72 h) in the absence of PTU and then directly plated on PPLO agar. The numbers of *M. pulmonis* recovered are expressed in terms of CFU/ml.

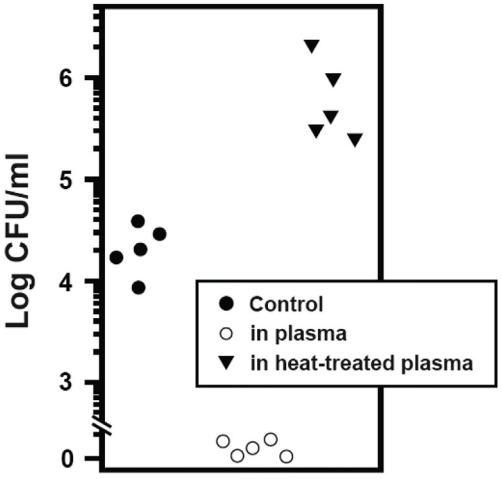


Fig. 2. *M. pulmonis* proliferated in heat-treated plasma. 1×10^5 CFU of *M. pulmonis* cultured in 20% naïve (○) or heat-treated plasma (▼) for 9 h at 36 °C. Control (●) indicates samples directly plated in insect saline without cultivation with plasma. The experiments were repeated five times and numbers of *M. pulmonis* recovered are expressed in CFU/ml.

significance of data was evaluated using student's *t*-test.

2.4. Antibacterial activity of plasma

A colony count assay was performed to determine the antibacterial activity of plasma induced by immune challenge with insect saline, *S. aureus* (*S.a*), *E. coli* (*E.c*) or *M. pulmonis* (*M.p*). After overnight cultivation, immunized plasma was collected from saline-, *S.a*-, *E.c*- or *M.p*-injected *T. molitor* larvae in the presence of PTU. Eighty microliter (1×10^6 CFU) of each bacterial suspension was mixed with 20 µl aliquots of each immunized plasma and then incubated for 30 min at 36 °C. Following incubation, 20 µl aliquots of the sample mixture (100 µl) were plated on TSB agar for bacteria or on PPLO agar for mycoplasma (either directly or after dilution). In addition, we tested the anti-mycoplasma activity of naïve (non-immunized) or heat-treated plasma. Naïve plasma was prepared in the absence of PTU or heat-treated for 30 min at 56 °C, after which it was centrifuged and the pellet removed. Mycoplasma suspension (80 µl) and naïve/heat-treated plasma (20 µl) were mixed and incubated for 9 h. Twenty µl from each sample was then plated on PPLO agar. Viable colonies were counted after incubation for 10 days in a humid chamber.

2.5. Isolation of antimicrobial peptides (proteins) from T. molitor plasma

Plasma was obtained in the presence of PTU from larvae immunized by a mixture of *S. aureus*, *E. coli*, and *M. pulmonis* was mixed with an equal volume of 10% acetic acid and agitated overnight at 4 °C. Following centrifugation for 30 min at 15,000 *g*, the supernatant was subjected to C_{18} reversed-phase HPLC (Vydac 214TP54). After washing the column with water containing 0.1% trifluoroacetic acid for 10 min, fractions were eluted with a linear gradient of 0–60% acetonitrile containing 0.1% trifluoroacetic acid for 60 min at a flow rate of 1 ml/min. The eluted fractions were collected at intervals of 1 min and each fraction was then tested for its antibacterial activity by an ultrasensitive radial diffusion assay using *S. aureus* and *E. coli* as previously described (Shin et al., 2010). Fractions exhibiting antibacterial activity were pooled and then dried in a Speed-Vac centrifugation system (Hanil Science, Korea). After the dried sample was resuspended in acidified water, its protein concentration was adjusted to 50 µg/ml via the bicinchoninic acid method (Pierce, Rockford, USA).

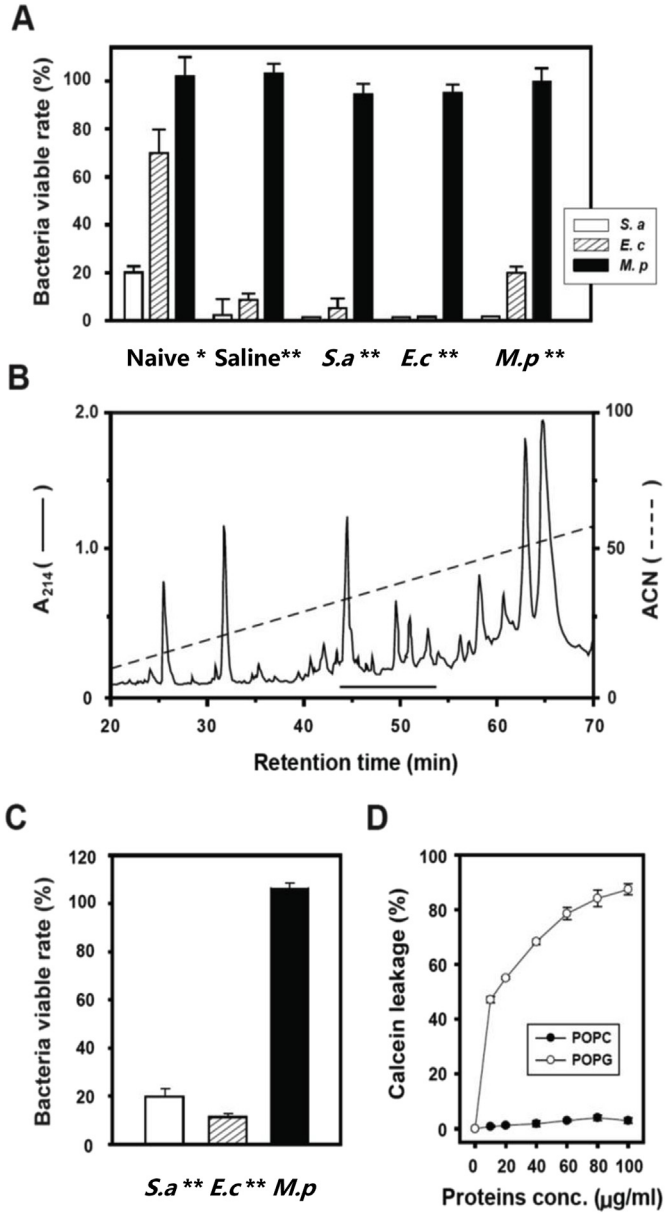


Fig. 3. AMPs of immunized *T. molitor* larvae exerted antibacterial activity against *S. aureus* and *E. coli*, but not against *M. pulmonis*. (A) Antibacterial activity of plasma induced by immune challenge was evaluated by colony count assay. Results are expressed as the survival % of the test sample relative to the control sample that was incubated in buffer. (B) RP-HPLC profile of the immunized *T. molitor* larvae hemolymph by bacteria mixture. Antibacterial fractions were determined by a radial diffusion assay against *S. aureus* and *E. coli*. Active fractions marked by a solid line on the bottom of profile were pooled. Then it was dried and resuspended in acidified H_2O (0.01% acetic acid) to use for following experiments. (C) Twenty µl of antibacterial fraction (50 µg/ml) obtained from HPLC was subjected to colony count assay for antibacterial activity against three types of bacteria. Data are expressed as in Fig. 3A. (D) Percent leakage from the calein-entrappted liposomes consisting of POPC or POPG. The maximum level of fluorescence (100%) was determined by complete lysis of liposomes treated with Triton X-100. The zero level corresponded to liposome fluorescence in the absence of protein. The data shown are the mean values of three independent experiments. Bars represent the standard errors of the means. Statistical significance was evaluated using ANOVA (*$P < 0.05$; **$P < 0.01$).

2.6. Preparation of liposome and calcein leakage assay

Liposomes were prepared and a calcein leakage assay was

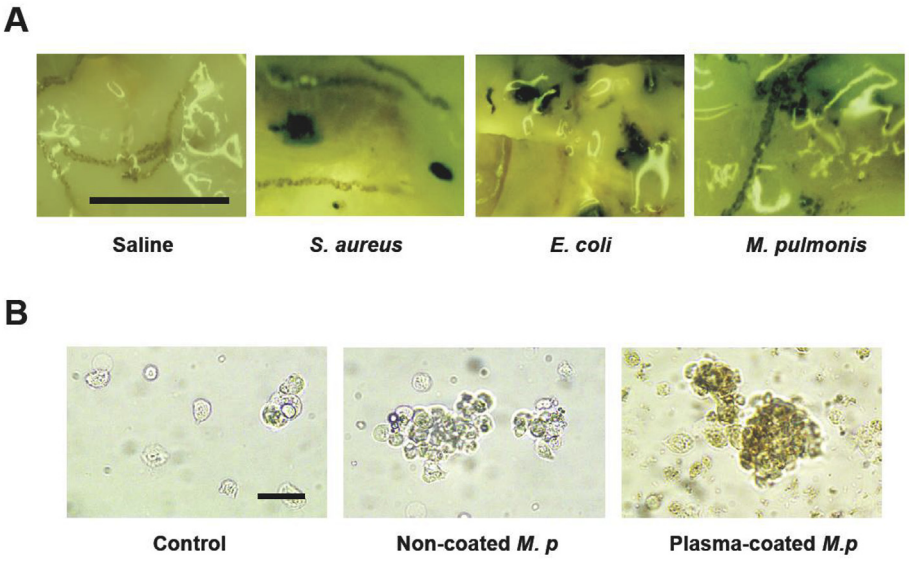


Fig. 4. Tm-SPH1 participates in nodule formation by mycoplasma infection. (A) *T. molitor* larvae challenged by injecting the bacteria into the hemocoel. At 12 h after injection, melanized nodules were found inside of the larva. Bar = 1 mm. (B) Hemocytes were incubated in Grace's insect medium in the presence of plasma coated or non-coated *M. pulmonis* and then observed microscopically. Dopamine substrate was added to all samples. Limited bacterial aggregation appeared in hemocytes treated with non-coated mycoplasmas. Developing melanizaion and nodule formation was obvious in the hemocyte sample treated with plasma-coated mycoplasmas. Bar = 20 μm. (C) SDS-PAGE analysis of plasma proteins extracted from the surface of *M. pulmonis* following the *in vitro* binding assay. Lane 1, standard marker; lane 2, control binding assay performed without plasma; lane 3, binding assay performed with plasma. The major band indicated by an arrow was transferred to PVDF membrane, and its N-terminal amino acid sequence was determined.

performed according to a previously described procedure (Chongsiriwatana and Barron, 2010). In this experiment, the following phospholipids were used: phosphatidylcholine (POPC), phosphatidylglycerol (POPG), cardiolipin, and sphingomyelin. Calcein-entrapped liposomes consisting of POPC or POPG were obtained by elution with 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl on a Sephadex G-50 column. In the calcein leakage assay, 20 μl of vesicle solution (final concentration 50 μM) in a 96-well microtiter plate (SPL, 30296) were mixed with 80 μl of a dilution of the protein samples in buffer. The fluorescence intensities of calcein were measured using a multi-mode microplate reader (BioTek, USA) with excitation and emission values of 495 and 575 nm, respectively. The percent dye-release was evaluated by the following equation: leakage (%) = $(F - F_0)/(F_{max} - F_0) \times 100$, where F_0 was the fluorescence intensity of the control vesicle and F and F_{max} were the fluorescence intensities achieved by samples and 1% (v/v) Triton X-100, respectively.

2.7. Microscopic observation for nodule formation and flow cytometry analysis for phagocytosis

For observation of *in vivo* nodule formation, 2 μl of bacteria suspension (1×10^6 CFU/larva) or insect saline was injected into the hemocoel. At 12 h after injection, larvae were anesthetized on ice, after which the hemocoel was exposed for photography. To observe *in vitro* nodule formation caused by mycoplasma, *M. pulmonis* cells (1×10^8 CFU) were first coated with plasma via incubation with 10 μl naïve plasma for 30 min at 36 °C. Plasma-coated mycoplasma was then washed three times and finally suspended with 1 ml of insect saline. Ninety microliters of Grace's Insect Media (GIM) containing hemocytes (5×10^6 cells) were pre-incubated with 10 μl of plasma-coated or non-coated mycoplasma suspension for 10 min, after which 100 μl of 2 mM dopamine hydrochloride (Sigma, H8502) dissolved in 20 mM Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$ was added. For a negative control, hemocytes were incubated with dopamine solution without mycoplasma. After incubation for 1 h, the sample was examined under light microscopy. Additionally, the phagocytosis of bacteria by hemocytes was evaluated via flow cytometry analysis with a Guava EasyCyte flow cytometer and analyzed by Guava InCyte software (Millipore, USA). For fluorescence activated cell sorter analysis, bacteria were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, 15710) in insect saline for 4 h at 37 °C and then bacteria was labeled with fluorescein isothiocyanate (FITC) in accordance with the procedure described previously (Costa et al., 2005). Fixed bacteria were then coated with plasma as described above. FITC-labeled bacteria (1×10^6 CFU) coated with plasma were mixed with hemocytes (1×10^5 cells) in a total volume of 100 μl of GIM. After 1 h incubation at 30 °C, to quench of the extracellular fluorescence, the samples were treated with 100 μl of 0.4% trypan blue for 10 min. After washing with GIM, 5000 hemocytes of each sample were counted. Data were obtained from three independent experiments and expressed as the mean values ± standard deviations.

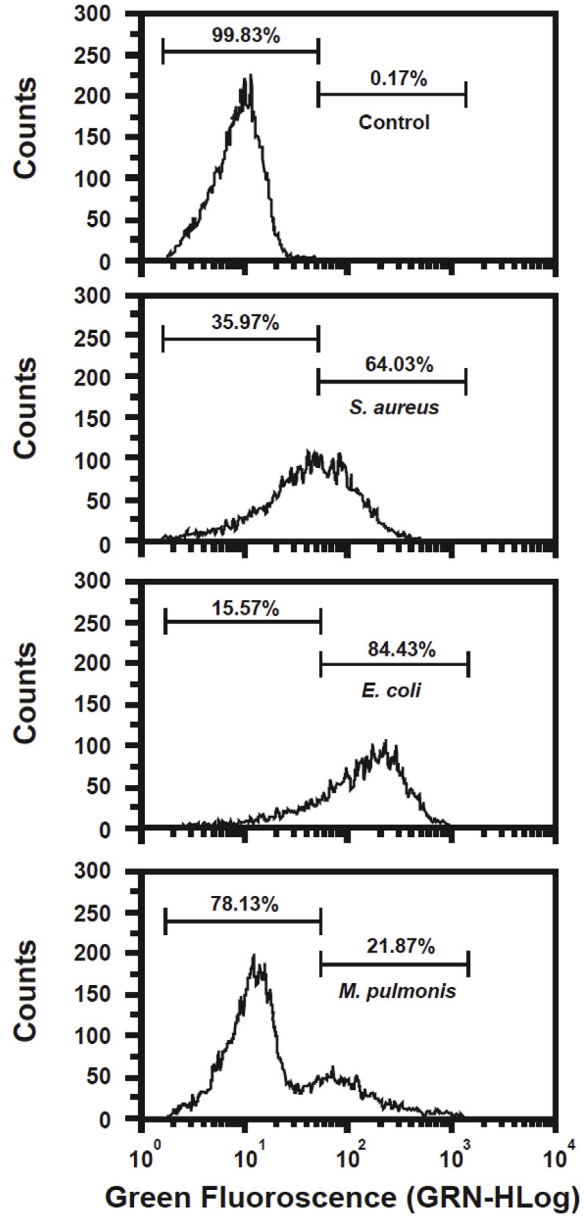


Fig. 5. *M. pulmonis* is resistant to phagocytosis by *T. molitor* hemocytes. Flow cytometry analysis for phagocytosis of *S. aureus*, *E. coli*, or *M. pulmonis* by hemocytes is shown in each panel. Hemocytes that did not incubated with bacteria were measured and indicated as a control (the first panel). The phagocytosis of *M. pulmonis* by hemocytes is much less active as compared with data obtained from experiments performed with *S. aureus* and *E. coli*.

2.8. Assay for phenoloxidase (PO) activity

A PO assay was carried out according to a previously published method (Lee et al., 2000). Briefly, paraformaldehyde-fixed bacteria coated with plasma were prepared as described above. To determine the PO activity induced by plasma-coated or non-coated bacteria, 10 μl of each bacteria sample (1×10^6 CFU) was mixed with 90 μl of insect saline. Alternatively, isolated hemocytes (5×10^6 cells) were added to the insect saline containing bacteria samples. Liposome samples were prepared as described above without calcein. Ten microliters of liposomes (20 μg) were incubated with 90 μl of naïve plasma sample containing 89 μl of insect saline for 15 min at 30 °C. All reactions were started by adding 100 μl of 2 mM dopamine and then incubated for 30 min at 30 °C. The increase in absorbance at 490 nm was measured using a microplate reader (Sunrise, Australia).

2.9. Assay for bactericidal activity by proPO activation or phagocytosis

Bactericidal effects caused by proPO activation on plasma-coated or non-coated bacteria were investigated in the absence of hemocytes. Ten microliters of plasma-coated or non-coated bacteria were mixed with 90 μl of insect saline, after which the mixture was incubated with 100 μl of 2 mM dopamine for 1 h at 36 °C. Next, 20 μl aliquots of the sample mixture were plated on TSB or PPLO agar, either directly or after dilution. Viable colonies were counted after incubation for 18 h (*S. aureus and E. coli*) or 10 days (*M. pulmonis*) at 36 °C. For a control, each bacteria sample was subjected to the same procedure without addition of dopamine. Data were represented by the percent of recovered CFU to CFU in the control sample. In addition, we examined the antibacterial effect caused by *T. molitor* hemocytes or RAW264.7 cells. Bacteria samples were mixed with hemocytes (1×10^7 cells) or RAW264.7 (5×10^5 cells) and incubated for 1 h at 36 °C in 50% GIM or RPMI, respectively. As a control, each bacteria sample was incubated with 50% GIM or RPMI in the absence of hemocytes or RAW264.7 under identical conditions. Recovered colonies were enumerated and data were generated by the same method as described above.

2.10. Identification of M. pulmonis binding protein

Paraformaldehyde-fixed *M. pulmonis* (1×10^9 CFU) were incubated with 5% (w/v) bovine serum albumin (BSA) in insect saline for 1 h at 37 °C. The sample was then centrifuged for 10 min at 2000 × *g* and 4 °C, after which the resultant pellet was suspended in 1 ml of insect saline containing 1% BSA and 100 μl of plasma. Next, the mixture was incubated for 1 h in an end-to-end rotator at 37 °C. Following centrifugation, the pellet was washed five times with insect saline containing 0.1% (v/v) Tween-20. Plasma proteins bound to the surface of *M. pulmonis* were detached using 50 μl of 0.1 M Glycine-HCl (pH 2.5) containing 150 mM NaCl and then subjected to brief centrifugation, after which the supernatant was subjected to 12% SDS-PAGE. The gel was stained with Coomassie blue and a duplicate gel was employed for electro-transfer onto a polyvinylidene-difluoride membrane. The membrane was stained with amido black and a major band with a molecular mass of about 45 kDa was excised and subjected to sequencing using an Applied Biosystem 476 A automated protein sequencer to determine the N-terminal amino acid sequence.

3. Results

3.1. Insecticidal effect of mycoplasma and its survival in insect hemocoel

In order to examine the insecticidal activity of mycoplasma, predetermined numbers of *M. pulmonis* were injected into the hemocoels of larvae. As shown in Fig. 1A, no dead larvae were found upon injection of 10^5 or 10^6 CFU. Injection of 10^7 CFU/larva resulted in a 10% decrease in survival until day 5. Evaluation of the effects of the control bacteria, *S. aureus* and *E. coli*, revealed an insecticidal rate of over 70% and 90%, respectively, when 10^7 CFU/larva were introduced (data not shown). These results indicate that *M. pulmonis* had low virulence relative to the control bacteria and/or was effectively suppressed by the immune system of *T. molitor*. We also investigated the survival of mycoplasma in the insect hemocoel by enumerating mycoplasma recovered at predetermined times after injection. The number of mycoplasma recovered from hemolymph decreased in a time-dependent fashion, and no mycoplasma was detected after 48 h of incubation (Fig. 1B). This finding led to doubt whether the changes might be due to starvation of mycoplasma in the insect hemolymph instead of the insect immune response against mycoplasma. Therefore, mycoplasma was cultured in the plasma (cell-free hemolymph) of *T. molitor* in the absence of growth media. Under these conditions, mycoplasma successfully proliferated in buffer containing heat-treated plasma (Fig. 2), while their numbers decreased significantly when cultured in the

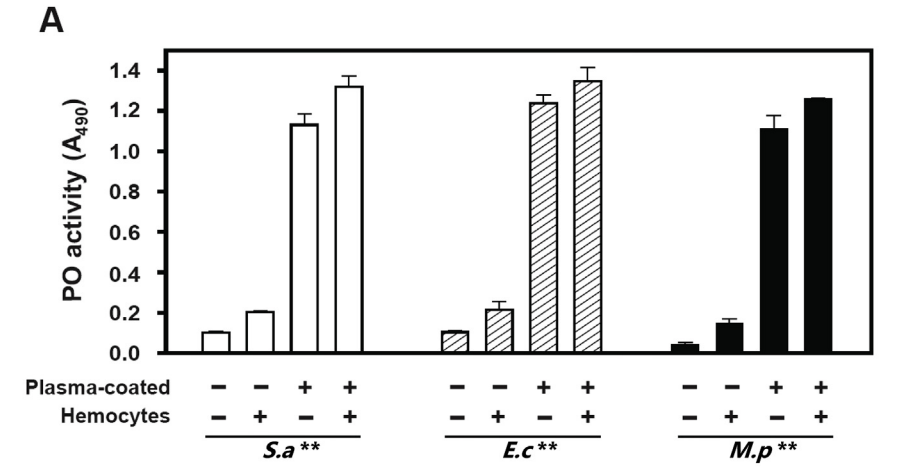
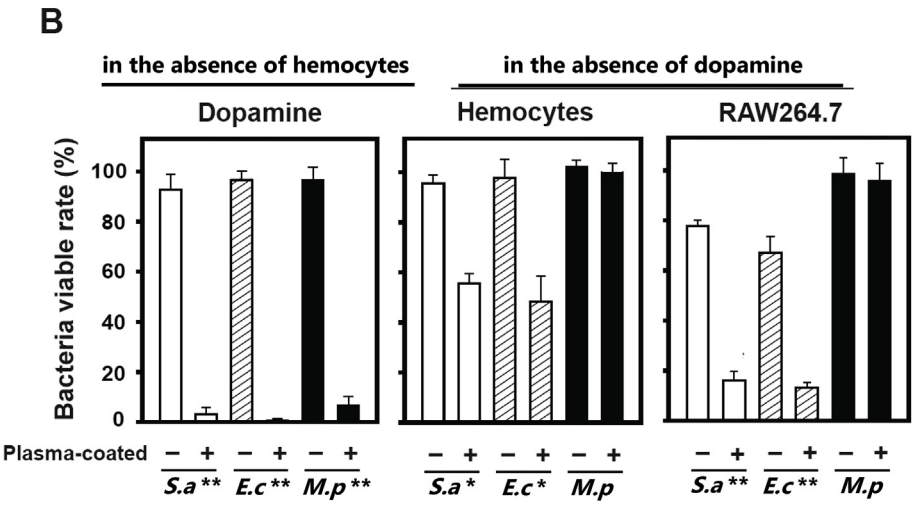


Fig. 6. Effect of PO activity (A) and bacterial survival (B) by plasma coated *M. pulmonis*. (A) Fixed bacteria were coated with naïve plasma and their activity for proPO activation was measured using dopamine substrate, as described in the Experimental Procedures section. Alternatively, hemocytes were added into the plasma coated or non-coated bacteria. Symbol '-' means 'no treatment with plasma and no addition of hemocytes'; symbol '+' means 'treatment with plasma and addition of hemocytes'. Statistical significance was evaluated using ANOVA (*$P < 0.05$; **$P < 0.01$). (B) Bactericidal effects caused by proPO activation or phagocytosis were evaluated by colony count assay. Symbols '-' and '+' mean the sample was not treated and treated with plasma, respectively. Data were obtained from three independent experiments and statistical significance was evaluated using student's *t*-test (*$P < 0.05$; **$P < 0.01$).



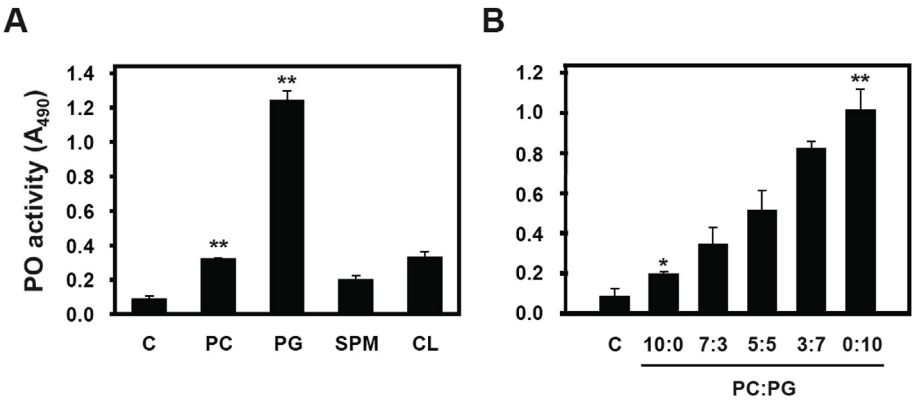


Fig. 7. POPG in membrane phospholipid activated proPO cascade. Naïve plasma was collected from the three larvae in the absence of PTU. For the control, 1 μl of plasma was incubated with insect saline. Each liposome (20 μg) was incubated with plasma for 15 min at 30 °C, after which the PO activity was measured using dopamine substrate as described in the Experimental Procedures. In the case of (B), liposomes were prepared with POPC and/or POPG. The molar ratio (PC:PG) of liposomes was 10:0, 7:3, 5:5, 3:7 and 0:10. Data were obtained from three independent experiments and statistical significance was evaluated using student's *t*-test (*$P < 0.05$; **$P < 0.01$).

presence of intact plasma. Taken together, these results indicate that the removal of mycoplasma introduced into the hemocoel was attributed to an anti-mycoplasma effect mediated by heat-labile components occurring in plasma, but not starvation.

3.2. Resistance of mycoplasma to attacks by antimicrobial peptides (proteins) in T. molitor plasma

Immunized plasma was obtained from larvae challenged with insect saline or each of three bacteria. We next investigated the anti-mycoplasma activity of immunized plasma by colony count assay after short-term incubation (30 min). A short time was adopted for incubation of plasma with bacteria because AMPs are known to exert their activity within 10 min. As shown in Fig. 3A, all types of immunized plasma showed strong antibacterial activity against two control bacteria. In contrast, all plasma samples had almost no effect on the viability of *M. pulmonis* during incubation for 30 min. These results suggested that mycoplasma were resistant to AMPs occurring in the plasma of immunized *T. molitor*. To further verify this assumption, AMPs were isolated from the plasma of *T. molitor* larvae immunized with a bacterial mixture of *S. aureus*, *E. coli* and *M. pulmonis* (Fig. 3B). Fractions showing antibacterial activity against *S. aureus* and *E. coli* in a radial diffusion assay were pooled and employed for colony count assays to evaluate the anti-mycoplasma activity. As a result, the sample was found to be

incapable of killing *M. pulmonis*, although it showed potent antibacterial activity against *S. aureus* and *E. coli* (Fig. 3C). In a consecutive experiment, the pooled fraction sample was tested for its binding to two major phospholipids (POPC and POPG) of mycoplasma membrane based on the previous finding that the anti-mycoplasma activity of AMPs mainly depends on the binding for POPC (Park et al., 2013). As shown in Fig. 3D, analyses using calcein-entrapped POPC or POPG liposomes revealed that the sample induced leakage of calcein from the POPG liposome in a dose-dependent manner. In contrast, when liposomes consisting of POPC were treated with the sample, little or no calcein leakage was detected. These results indicated that AMPs in plasma from immunized *T. molitor* did not interact with POPC of mycoplasma membrane lipids, and therefore exhibited no anti-mycoplasma activity.

3.3. Effect of in vivo and in vitro nodule melanization on mycoplasma infection and phagocytosis

To identify an insect immune response against mycoplasma infection, we observed the inside of larvae under the dissecting microscope at 12 h after injection of *M. pulmonis*, or of S. *aureus* and *E. coli* as positive controls. As shown in Fig. 4A, several melanized nodules were found inside of the larvae infected with mycoplasma, as well as in that of larvae infected with the two positive controls. These results indicated that the immune response against mycoplasma infection included nodulation mediated by hemocytes and melanization induced by activation of the proPO cascade. Therefore, we investigated the *in vitro* nodule melanization by observation under a light microscope after incubation of mycoplasma with hemocytes in the presence of substrate (dopamine) for PO (Fig. 4B). While nodule formation was found in both samples containing intact and plasma-coated mycoplasma, the melanization of nodules was only detected in the mixture of plasma-coated mycoplasma and hemocytes. These results suggested that the surface of mycoplasma might be bound by plasma components required for proPO activation when mycoplasma was incubated with plasma. To verify this postulation, we characterized proteins detached from the surface of mycoplasma after its incubation with plasma. We found a protein that was completely homologous with the sequence of N-terminal 15 amino acids of an active form of serine protease homolog 1 (Tm-SPH1) (Fig. 4C), which was previously demonstrated to be an essential protein for triggering activation of the *T. molitor* proPO system (Lee et al., 2002; Kan et al., 2008). In addition, we investigated the phagocytosis of *M. pulmonis* by *T. molitor* hemocytes via the flow cytometric analyses. As shown in Fig. 5, FITC-labeled *M. pulmonis* (21.87 ± 2.80%) was much less internalized into hemocytes than *S. aureus* (64.03 ± 7.30%) with $P < 0.05$ and *E. coli* (84.43 ± 3.80%) with $P < 0.001$. Mean differences were tested by student's *t*-test. These results suggest thst the phagocytosis is not effective in the removal of mycoplasma that was introduced into the insect hemocoel.

3.4. Relationship between proPO activation and viability of mycoplasma

The effect of proPO activation on the viability of mycoplasma was investigated under a variety of conditions. We first measured the PO activity induced by mycoplasma. As in the case of the two control bacteria, plasma-coated mycoplasma also showed to lead a high PO activation (Fig. 6A), indicating that the surface of mycoplasma was equipped with the plasma components required for PO activity. In addition, hemocytes slightly enhanced the PO activity, regardless of the type of bacteria. In consecutive experiments, we assessed the survival of mycoplasma upon proPO activation and examined whether the viability of mycoplasma was affected by insect hemocytes and murine macrophages (RAW264.7 cells) in the absence of PO activity originated from the *T. molitor* plasma (Fig. 6B). The results revealed that the viable rate of mycoplasma was significantly decreased when PO activity was ensured, as in the case of the two control samples. In contrast, hemocytes and mouse phagocytes showed little or no effect on the viability of mycoplasma in the absence of PO activity, although the survival of the two control bacteria obviously decreased upon incubation with each of the two cells. Therefore, it was concluded that mycoplasma was killed by toxic intermediates generated by activation of proPO cascade, but that its viability was not affected by cellular immune reactions such as phagocytosis and nodule formation under the condition without PO activity. .

3.5. Activation of proPO cascade by phospholipids constituting mycoplasma membrane

We measured activation of the proPO cascade in *T. molitor* plasma by four kinds of phospholipids that were found to occur in the *M. pulmonis* membrane (Park et al., 2013). As shown in Fig. 7A, POPG led to significantly higher activation of proPO than the other three phospholipids. These findings were confirmed by experiments performed using liposomes consisting of POPC and/or POPG (Fig. 7B), which are known to be major phospholipids in the outer leaflet of the mycoplasma membrane lipid bilayer. These results indicate that higher levels of POPG present in the liposome lead to stronger activation of the proPO cascade. Overall, the activation of the proPO cascade in plasma was triggered by POPG occurring in the outermost lipid layer of the mycoplasma, thereby capable of exerting anti-mycoplasma activity.

4. Discussion

Mycoplasmas are widespread in nature, and many are pathogenic toward humans and animals. In addition, some are notorious contaminants of cell and tissue cultures. Mycoplasmas are evolutionary descendants of Gram-positive bacteria that are taxonomically separated from normal bacteria equipped with cell walls and belong to the class Mollicutes (*mollis*, soft; *cutis*, skin). These organisms lack cell walls, but possess distinct plasma membranes containing sterols acquired from their growth environment (Rottem, 2002; Kornspan and Rottem, 2012). When compared to other pathogenic bacteria with cell walls, mycoplasmas are not highly virulent, but tend to persist in their host for extended periods after infection. Therefore, mycoplasma-caused diseases generally follow a progressive chronic course with low mortality and high morbidity (Rosengarten et al., 2000). The pathogenic mechanisms of mycoplasmas have recently been gradually uncovered owing to great advances in genetic techniques (Jaffe et al., 2004; Li et al., 2015; Waites et al., 2017). However, general appreciation of the virulence potential of these microbes is still lacking. Moreover, the biochemical interactions between mycoplasmas and the host immune system are difficult to define owing to the unique simplicity of their cell envelope and the fastidious properties of their growth, including the overly long doubling time.

To date, limited information has been generated from kinetic studies of the host immune response against mycoplasma infections. It has been demonstrated that lipoproteins in the mycoplasma membrane play a critical role in the virulence and immune-modulatory effect of mycoplasmas (Chambaud et al., 1999; Browning et al., 2011). It is believed that most membrane-anchored lipoproteins are exposed to the extracellular milieu, and they appear to trigger a lympho-proliferative response in the host, which is a primary pathogenic mechanism of mycoplasma diseases. It has been found that lipoproteins contribute to this pathogenesis by modulating the production of a variety of cytokines and chemokines (Bodhankar et al., 2010; Browning et al., 2011). In addition, this immune-modulating activity of mycoplasmas was shown to be attributable to recognition of the surface lipoproteins by TLR-2/TLR-6 and subsequent activation of the corresponding signaling pathway (Muneta et al., 2003; McGowin et al., 2009; Shimizu, 2016). However, this immune response mediated by membrane lipoproteins is not considered a specific phenomenon observed only upon mycoplasma infection as there are a number of reports showing that membrane

lipoproteins are present in a wide range of normal wall-covered bacteria that are also associated with TLR-mediated inflammatory responses to bacterial infections (Lorne et al., 2010; Evavold and Kagan, 2018). Accordingly, it was suggested that elucidation of the specific interaction between mycoplasmas and the immune system might enable a better understanding of the pathological and immunological aspects of mycoplasma infections, which are distinctly different from those of infections with normal bacteria.

T. molitor larvae might be useful as a model insect in a variety of studies of microbial virulence and innate immune system functions since its defense systems, which include the proPO cascade (Cerenius et al., 2008; Kan et al., 2008), AMPs (Chae et al., 2012) and cellular immunity (Lemaitre and Hoffmann, 2007), have been well described to date. In this study, we showed that *T. molitor* larvae protected themselves from intruding mycoplasmas via activation of their proPO system, but neither AMPs nor cellular reactions such as phagocytosis and nodulation exerted their activity under conditions with no guaranteed PO activity. As shown in Fig. 6B, *M. pulmonis*, the causative agent of murine respiratory mycoplasmosis, was significantly resistant to phagocytosis by *T. molitor* hemocytes and RAW264.7 when compared to *E. coli* and *S aureus*. This anti-phagocytic property of *M. pulmonis* was consistently observed, regardless of pre-incubation with insect plasma for opsonization of mycoplasmas. *M. pulmonis* was previously shown to be resistant to attack by alveolar macrophages, which play a primary role in defense against respiratory infection with mycoplasma (Hickman-Davis et al., 1997). In addition, as in the case of this study, mouse alveolar macrophages were ineffective at killing *M. pulmonis*, even in the presence of anti-mycoplasma antibody for opsonization (Hickman-Davis et al., 1998). Furthermore, some studies reported that the anti-phagocytic effect of *M. pulmonis* could be attributed to two surface components, the variable surface antigen (Vsa) family of lipoproteins (Shaw et al., 2012) and the capsular exopolysaccharide (Shaw et al., 2013). The Vsa protein was shown to function as an anti-phagocytic factor by inhibiting interactions of mycoplasmas with alveolar macrophages. *M. pulmonis* was also shown to produce an exopolysaccharide, referred to as EPS-I, which played an anti-phagocytic role via inhibition of the binding of *M. pulmonis* to alveolar macrophages. Therefore, it was suggested that the resistance of *M. pulmonis* to phagocytosis by *T. molitor* hemocytes shown in this study might also be due to Vsa proteins and/or EPS-I on the surface *of M. plumonis*.

To date, the proPO system of insects has been extensively studied, and several protein components were shown to be involved in activation of the proPO cascade (Kim et al., 2008; Yu et al., 2010). As a result of activation of the proPO cascade, melanin was formed on the surface of bacteria, which caused a strong bactericidal effect. Investigation of activation of the proPO cascade in *T. molitor* revealed that PO and an active form of SPH1 were generated by Spatzel-processing enzyme upon bacterial infection, and that both were co-localized on melanin-concentrated areas of the surface of bacteria (Kan et al., 2008). As a cofactor of PO, the active form of SPH1 was shown to play an indispensable role in PO activity. Accordingly, the presence of SPH1 on the surface of intruding microbes might guarantee activation of proPO cascade in *T. molitor*. We attempted to confirm that the proPO cascade was activated upon intrusion of *M. pulmonis* into the hemocoel of *T. molitor* based on molecular analyses. The results revealed that an active form of SPH1 was present on the surface of mycoplasma (Fig. 4C), supporting activation of the proPO system by mycoplasma as in the case of normal bacterial infection. It is well known that cell wall components of normal wall-covered bacteria, such as peptidoglycan and lipopolysaccharide, trigger activation of the proPO cascade in insect hemolymph (Cerenius and Soderhall and Cerenius, 1998; Kan et al., 2008). However, it is not clear how mycoplasmas, which have plasma membranes directly exposed to the exterior, trigger activation of the insect proPO system. Experiments performed using liposomes consisting of phospholipids found in the mycoplasma membrane revealed that POPG made a significantly greater contribution to activation of the proPO cascade than other membrane phospholipids such as POPC, sphingomyelin and cardiolipin. These findings suggest that POPG occurring in the outer leaflet of the membrane lipid bilayer might play a critical role in proPO activation upon mycoplasma infection, unlike in the case of infections with normal bacteria containing membrane lipids that were covered with solid cell walls.

Based on the results shown in Fig. 3, it was assumed that inducible AMPs of *T. molitor* were ineffective at killing mycoplasmas upon mycoplasma infection. AMPs were previously shown to have some disparity in their antimicrobial activities against mycoplasmas (Park et al., 2013). While some AMPs (LL-37 and HG1) were active at killing mycoplasmas, others (magainin, cecropin and polymyxin B) were found to have little or no anti-mycoplasma activity. AMPs with anti-mycoplasma activity were shown to kill mycoplasmas by rupturing their plasma membrane within a short period of time (30 min), which was similar to the mode of action against normal bacteria equipped with cell walls (Shai, 2002). In addition, it was shown that the anti-mycoplasma activity of AMPs depends on the affinity for POPC occurring in the outer leaflet of the membrane lipid bilayer (Park et al., 2013). In parallel, we also found that AMPs of *T. molitor* were not capable of attacking the mycoplasma membrane (Fig. 3C) as they had little or no affinity for POPC (Fig. 3D). Taken together, the results of this study indicate that POPG and POPC occurring in the outer layer of the mycoplasma membrane lipid bilayer were associated with activation of the proPO system and the anti-mycoplasma activity of AMPs, respectively.

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Declarations of interest

None.

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