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# The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*

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Among *Legionella* species, which are recognized to be pathogenic for humans, *L. gormanii* is the second prevalent causative agent of community-acquired pneumonia after *L. pneumophila*. Anti-*L. gormanii* activity of *Galleria mellonella* hemolymph extract and apolipophorin III (apoLp-III) was examined. The extract and apoLp-III at the concentration 0.025 mg/ml caused 75% and 10% decrease of the bacteria survival rate, respectively. The apoLp-III-induced changes of the bacteria cell surface were analyzed for the first time by atomic force microscopy. Our studies demonstrated the powerful anti-*Legionella* effects of the insect defence polypeptides, which could be exploited in drugs design against these pathogens.

**Key words**: *Legionella gormanii*, *Galleria mellonella*, apolipophorin III, Atomic Force Microscopy

## INTRODUCTION

Currently, members of the family *Legionellaceae* comprise 58 described species that are highly successful in colonizing natural aquatic environments (Euzeby, 2013). A particular hallmark of these bacteria is their dual host system allowing intracellular growth in protozoa (from the genera *Acanthamoeba*, *Naegleria*, *Hartmanella*) and in human alveolar macrophages during infection (Fields *et al.*, 2002). The ability to survive and replicate inside unicellular organisms contributed to the acquisition of virulence factors that enable *Legionella* to overcome the antimicrobial activities of human macrophages. The clinical manifestations of *Legionella* infections are primarily related to the respiratory tract. The most common presentation is acute pneumonia, which varies in severity from mild illness to fatal multilobar pneumonia. *Legionella* is widely recognised as an important source of community- and hospital-acquired pneumonia. The second form of a respiratory illness is called Pontiac fever, which is a flu-like infection (Palusinska-Szysz & Cendrowska-Pinkosz, 2009). The first described *Legionella* species, *L. pneumophila*, is unceasingly the dominating species among clinical isolates, whereas *L. gormanii* is the second prevalent causative agent of community-acquired pneumonia (Lode, 1987). *Legionella* infections are difficult to treat because of their intracellular localization in phagocytic cells. Moreover, bacteria released from *A. castellanii* are more resistant to chemical disinfectants and antibiotics used to treat pneumonia in comparison with those residing out-

side amoebae in the environment or laboratory cultured on *Legionella* artificial medium. A combination of fluoroquinolones with macrolides is an effective method of Legionnaires' disease treatment. However, the mortality rate among patients with hospital-acquired pneumonia is 14%, and with community-acquired pneumonia ranges from 5 to 10% (Benin *et al.*, 2002; Palusinska-Szysz, 2011). Therefore, the development of new antibacterial agents for *Legionella* infections is urgently needed.

A rich source of natural defence peptides (antimicrobial peptides; AMPs) with different biochemical properties and antimicrobial activity is insect's hemolymph (Bulet *et al.*, 2004; Bulet & Stöcklin, 2005). Due to amino acid composition, amphipathicity, cationic charge, and molecular size, AMPs can interact with microbial cell membranes forming toroidal or barrel-stave pores, or micelles composed of peptides and the membrane phospholipid molecules. Disturbing a proper structure of a cell membrane leads to depolarization, increased permeabilization, and even membrane fragmentation, which results in death of the invading microbes (Bulet *et al.*, 2004). These properties and selective toxicity towards some pathogens make them the patterns for designing drugs alternative to antibiotics.

A great arsenal of defence peptides of different biochemical and antimicrobial properties has been described in *Galleria mellonella* immune hemolymph. In addition, lysozyme and apolipophorin III (apoLp-III) play important role in the insect immune response against bacteria and fungi (Hultmark, 1996; Weers & Ryan, 2006; Cytryńska *et al.*, 2007; Brown *et al.*, 2008; 2009; Zdybicka-Barabas *et al.*, 2013). ApoLp-III, an abundant hemolymph protein, is involved in lipid transport and immune reactions. Because of lipopolysaccharide (LPS), lipoteichoic acid (LTA), and β-1,3-glucan binding ability, the protein is considered as an important pattern recognition receptor (Halwani *et al.*, 2000; Pratt & Weers, 2004; Whitten *et al.*, 2004; Zdybicka-Barabas & Cytryńska, 2013). The defence proteins and peptides isolated in our laboratory from immune hemolymph of *G. mellonella* larvae exhibit antimicrobial activity against different fungi, Gram-positive and Gram-negative bacteria

(Cytryńska *et al.* 2007; Zdybicka-Barabas & Cytryńska, 2011; Zdybicka-Barabas *et al.*, 2012a; 2012b; 2013). Recently, the greater wax moth *G. mellonella* has been successfully used to study pathogenesis and infection by different human pathogenic bacteria and fungi, including *L. pneumophila* (Harding *et al.*, 2012; 2013a; 2013b). In our previous study, anti-*L. dumoffii* activity of *G. mellonella* defensin and apoLp-III has been documented indicating promising potential of the insect defence factors in fighting *Legionella* spp. (Palusinska-Szysz *et al.*, 2012).

In order to test antimicrobial activity of *G. mellonella* defence compounds against other species of *Legionella*, the effects of hemolymph methanolic extracts and the purified apoLp-III on *L. gormanii* cells were investigated. In addition, the apoLp-III-induced changes of the bacteria cell surface topography and properties were imaged and analyzed by atomic force microscopy (AFM). Our study is an attempt at assessment of the potential of the new agents in elimination of *L. gormanii*.

## MATERIALS AND METHODS

**Microorganisms and growth conditions**. *L. gormanii* strain NCTC 11401 (Health Protection Agency Culture Collections, Salisbury, UK) was cultured on buffered charcoal-*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) yeast extract agar plates (BCYE; pH 6.9) (Oxoid, Basingstoke, UK) for 72 h at 37°C in a humidified incubator (5% $CO_2$) (Feeley *et al.*, 1979). *Micrococcus luteus* ATCC 10240 and *Escherichia coli* D31 (Boman *et al.*, 1974) were grown in 2.5% Luria-Bertani (LB) medium at 28°C and 37°C, respectively.

**Insects immunization and preparation of hemolymph methanolic extracts**. The larvae of *G. mellonella* (Lepidoptera: Pyralidae) were reared on honeybee nest debris (a natural diet) at 30°C in the dark. The immune challenge was performed by puncturing of the last instar larvae with a needle dipped into a pellet containing live *E. coli* D31 and *M. luteus* cells. The immune hemolymph was collected 24 h after the challenge. The methanolic extracts containing antimicrobial peptides and proteins below 30 kDa were prepared from the hemocyte-free hemolymph and deprived of lipids as described earlier (Cytryńska *et al.*, 2007). The protein concentration was determined by a Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

**Purification of apolipophorin III**. *G. mellonella* apoLp-III was purified from the immune hemolymph extract as described in our previous study (Zdybicka-Barabas *et al.*, 2013). Briefly, the freeze-dried hemolymph extract dissolved in 0.1% trifluoroacetic acid (TFA) was subjected to the HPLC chromatography using a Discovery Bio Wide Pore C18 4.6 mm × 250 mm column (Sigma-Aldrich, USA) and two buffer sets, A: 0.1% TFA (v/v), B: 0.07% TFA, 80% acetonitrile (v/v). A linear gradient from 30 to 70% of buffer B over 35 min and 1 ml/min flow rate was applied. The homogenous fraction containing apoLp-III was freeze-dried, subjected to weighting, redissolved in sterile deionized water, and stored at –80°C until use. The homogeneity and identity of apoLp-III was confirmed by SDS-PAGE electrophoresis (Schägger & von Jagow, 1987) and by N-terminal sequencing on an automatic protein sequencer (Procise 491, Applied Biosystems).

**Antimicrobial assays**. The activity of immune hemolymph extract and apoLp-III against *L. gormanii* was carried out using a colony counting assay as described previously (Palusinska-Szysz *et al.*, 2012). Briefly, 10 µl of a bacterial suspension (obtained by $2 \times 10^{-4}$ dilution of a suspension with $OD_{620} = 0.1$) was incubated without (control) or with the extract (final protein concentrations 0.025–0.8 mg/ml) or apoLp-III (final protein concentrations 0.025–0.2 mg/ml) at 37°C for 1 h. Then, the incubation mixtures were spread onto BCYE medium plates, incubated for 4 days at 37°C, and the number of the colony-forming units (CFU) was determined. The minimal inhibitory concentration (MIC) was defined at the concentration which yielded 95% inhibition of bacterial growth. The data were calculated from three independent experiments, each performed in triplicate.

**Atomic force microscopy imaging of *L. gormanii***. Forty µl of a water suspension ($OD_{620}=0.2$) containing the *L. gormanii* cells grown on BCYE medium were incubated without (control) and in the presence of purified *G. mellonella* apoLp-III (final concentration 0.2 mg/ml) at 37°C for 1 h. After centrifugation ($8\,000 \times g$, 10 min., 4°C) the bacteria were suspended in 5 µl of pyrogen-free water, applied on the surface of mica discs and allowed to dry overnight at room temperature.

*L. gormanii* cell surface imaging was carried out using NanoScope V AFM (Veeco, USA) in "PeakForce QNM" operation mode with a NSG 30 silicon tip (spring constant of 20N/m; NT-MDT, Russia) (Analytical Laboratory, Faculty of Chemistry, UMCS, Lublin, Poland). The data were analyzed with Nanoscope Analysis ver. 1.40 software (Veeco, USA). Two fields on each mica disc were imaged. The roughness values were measured over the entire bacterial cell surface on 3 µm×3 µm areas. The average surface root-mean-square (RMS) roughness was calculated from forty fields (265 nm×265 nm).

**Statistics**. Statistical analysis was performed using the Wilcoxon's paired test. The data were presented as ± standard deviation (SD) from three independent experiments.

## RESULTS AND DISCUSSION

The antibacterial activity of *G. mellonella* immune hemolymph extract against *L. gormanii* was evaluated. The results showed dose-dependent killing of *L. gormanii* cells by the extract (Table 1). The incubation of *L. gormanii* in the presence of the extract at the concentration 0.025 mg/ml for 1 h caused more than 75% decrease of the bacteria survival rate compared to the control,

**Table 1. The growth inhibition of *L. gormanii* by *G. mellonella* immune hemolymph extract.**

| Concentration of hemolymph extract (mg/ml) | Survival rate (%) |
|---|---|
| 0 | 100 (%) |
| 0.025 | 23.8 (±1.5) (%) |
| 0.05 | 15 (±0.6) (%) |
| 0.1 | 11 (±0.6) (%) |
| 0.2 | 7 (±0.6) (%) |
| 0.4 | 6 (±0.6) (%) |
| 0.8 | 5 (±0.6) (%) |

*L. gormanii* was incubated with the *G. mellonella* immune hemolymph extract at the concentrations of 0–0.8 mg/ml at 37°C for 1 h. The bacteria were then seeded on BCYE agar plates and the colonies were counted after four days incubation at 37°C in a 5% $CO_2$ atmosphere. Data were expressed as means ±S.D. of three independent experiments.
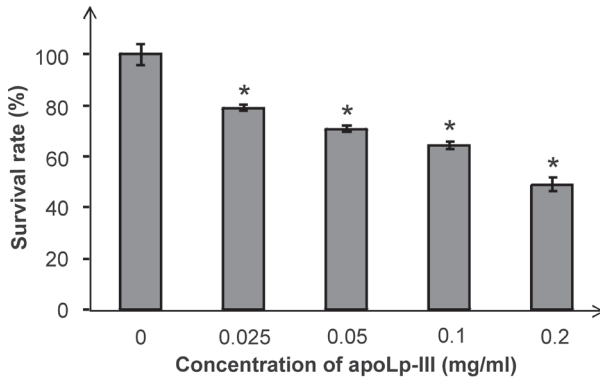
**Figure 1. The effect of *G. mellonella* apoLp-III on *L. gormanii* survival rate.**
The bacteria were incubated with the *G. mellonella* apoLp-III at the concentrations of 0–0.2 mg/ml at 37°C for 1 h. Cells were then plated on BCYE agar and the number of colonies was counted after four days incubation at 37°C in a 5% $CO_2$ atmosphere. Experimental results were mean ±S.D. of three independent experiments. The Wilcoxon's paired test was used for comparisons within in groups. *p* values *$p \leq 0.001$ were considered.

i.e. non-treated bacteria. The MIC value defined as the concentration yielding at least 95% inhibition of bacterial growth for the extract was determined to be 0.8 mg/ml. However, the extract used at the concentration 0.4 mg/ml reduced the bacteria survival rate to almost the same extent, i.e. by 94%. Recently, we have reported that antimicrobial proteins and peptides of the *G. mellonella* immune hemolymph extract inhibited *L. dumoffii* growth. When the extract was used at the concentration 0.4 mg/

ml, ca. 50% decrease of *L. dumoffii* survival rate was observed (Palusinska-Szysz *et al.*, 2012).

A main protein component of *G. mellonella* hemolymph extract is apoLp-III (Cytryńska *et al.*, 2007). Hence, the effect of apoLp-III on *L. gormanii* survival rate was studied. A colony counting assay using apoLp-III at the concentrations 0.025–0.2 mg/ml revealed that *L. gormanii* cells were sensitive to apoLp-III. The protein at the concentration 0.2 mg/ml caused ca. 50% decrease of the bacteria survival rate (Fig. 1). Interestingly, when apoLp-III was used at the concentration 0.025 mg/ml, the survival rate of *L. gormanii* decreased only by 10%, whereas the hemolymph extract at the same concentration caused 75% reduction of bacterial survivability (Fig. 1). The results indicated that although *L. gormanii* was susceptible to apoLp-III action, other proteinaceous (e.g. defence proteins and peptides) and non-proteinaceous compounds present in *G. mellonella* hemolymph extract were probably also involved in killing of the bacteria. Increasing of the hemolymph extract and apoLp-III concentrations above 0.8 mg/ml and 0.2 mg/ml, respectively, did not reduce further *L. gormanii* survival rate (data not shown). However, the explanation of this fact needs further investigations.

In our previous papers, usefulness of an atomic force microscopy for analysis of an influence of *G. mellonella* defence factors on bacterial and fungal cell surface has been demonstrated (Zdybicka-Barabas *et al.*, 2011; 2012a; 2012b; 2013). In this study, AFM was used for examining of the *G. mellonella* apoLp-III effect on *L. gormanii* cell surface. AFM imaging revealed that the surface of *L. gormanii* control cells was covered with small uniform granules (Figs. 2, 3). On the surface of some control cells few shallow depressions were also visible.
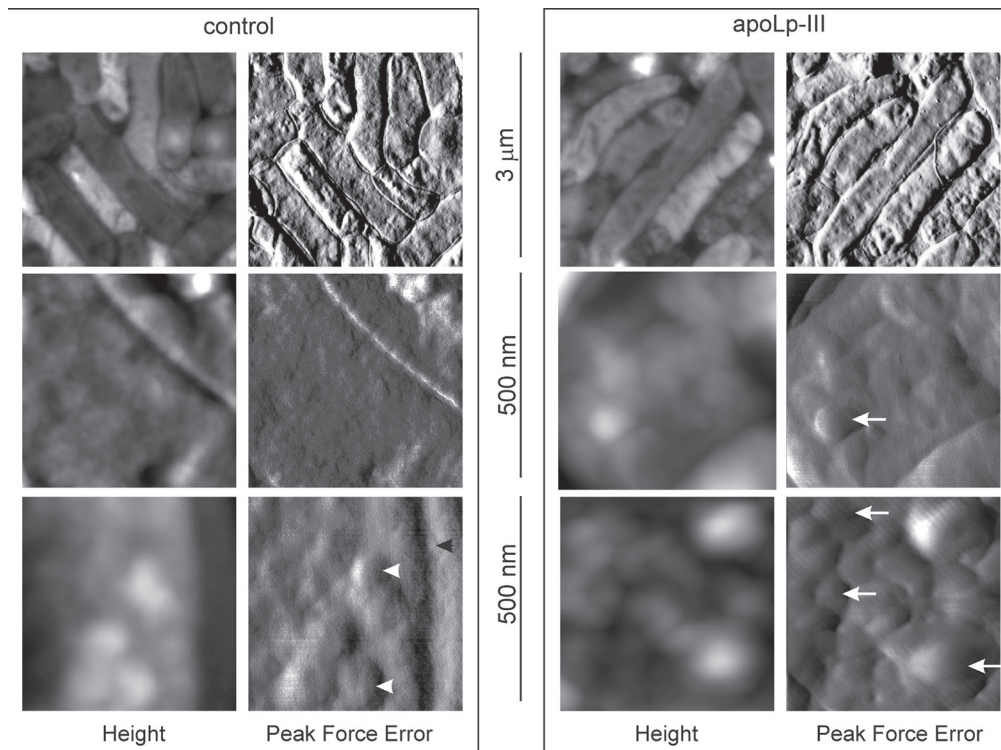


**Figure 2. The effect of *G. mellonella* apoLp-III on *L. gormanii* cell surface topography.**
The cells were incubated without (control) or in the presence of apoLp-III (0.2 mg/ml) at 37°C for 1 h and then imaged by AFM. The height and "peak force error" images are presented. The white and black arrowheads indicate granules and furrows, respectively, on the control cells surface. The white arrows mark bubble-like features appearing on apoLp-III-treated cells.
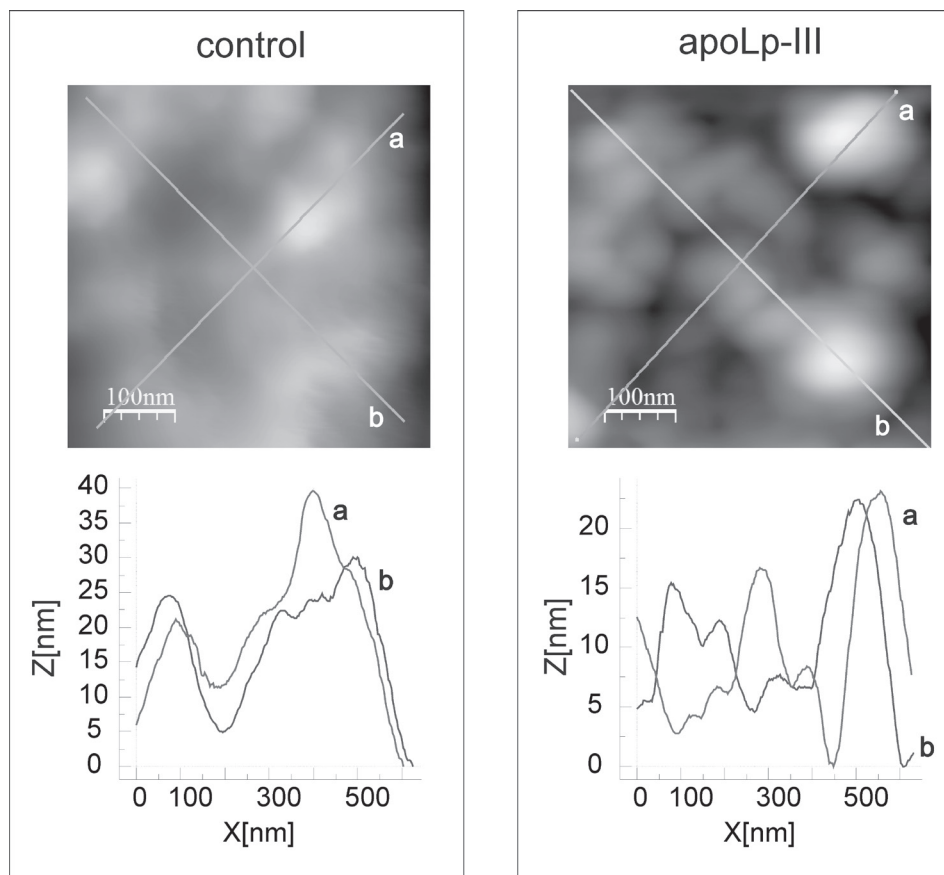
**Figure 3. Profile section analysis of *L. gormanii* cell surface.**
The cells were incubated without (control) or in the presence of apoLp-III (0.2 mg/ml) at 37°C for 1 h and then imaged by AFM. The upper panels present the height images of the cell surface. The bottom panels demonstrate the section profiles corresponding to the lines (**a**, **b**) shown in the height images.

In addition, the cells with uneven surface covered with furrows, some of them 10–20 nm deep and even 200 nm wide, were detected in the control samples. In contrast, the most of the apoLp-III-exposed cells were decorated with numerous rounded bubble-like features of different size, 15–20 nm in height and 100–200 nm in diameter (Figs. 2, 3). The alterations of the *L. gormanii* cell surface caused by apoLp-III were also reflected by 1.44-fold increase in cell surface roughness, the parameter used to describe the structural heterogeneity of the bacterial cell surface. The RMS roughness values for the control and apoLp-III-treated cells were calculated as 2.935 nm (±1.016) and 4.2305 nm (±1.003; $p$=0.00103), respectively. One of the reasons of the increase of the surface roughness could be rupturing of bacterial cell following the apoLp-III binding to LPS and phospholipids, which was reported by Pratt & Weers (2004).

Although both *L. dumoffii* and *L. gormanii* were sensitive to *G. mellonella* apoLp-III, the 8-fold lower concentration of apoLp-III was sufficient to exert the same bactericidal effect on *L. gormanii* in comparison to *L. dumoffii* (Palusinska-Szysz *et al.*, 2012). The high sensitivity of *L. gormanii* to apoLp-III in comparison with *L. dumoffii* indicates that different *Legionella* species could exhibit diverse susceptibility to the insect-derived antimicrobial factors, possibly reflecting differences in the cell surface properties.

Searching for antimicrobials effective against *L. gormanii* is especially important since this species was isolated from peadiatric cases. Moreover, antimicrobial therapy commonly used in empirical pneumonia treatment in young patients is not effective against *Legionella* spp. (Ephros *et al.*, 1989; Greenberg *et al.*, 2006).

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