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Midgut lysozymes of *Lucilia sericata* – new antimicrobials involved in maggot debridement therapy

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Abstract

Larvae of *Lucilia sericata* are used for maggot debridement therapy (MDT) because of their ability to remove necrotic tissue and eradicate bacterial pathogens of infected wounds. So far, very few antibacterial factors have been fully characterized (eg lucifensin). Using a molecular approach, some other putative antimicrobial compounds, including three novel lysozymes, have been previously identified and predicted to be involved in MDT. Nevertheless, data on lysozymes tissue origin and their functions have never been elucidated. Therefore, the aim of this study was to investigate the expression of three lysozymes in *L. sericata* and confirm their antibacterial effects within MDT. Moreover, we characterized the eradication process of bacteria within the digestive system of maggots and determined the role of lysozymes in this process. We found that three lysozymes are expressed in specific sections of the *L. sericata* midgut. Recombinant lysozymes displayed comparable antibacterial activity against *Micrococcus luteus*. Furthermore, the majority of Gram-positive bacteria were destroyed *in vivo* within the particular section of the *L. sericata* midgut where lysozymes are produced. Larval ingestion and subsequent eradication of wound pathogens during their passage through the intestine of maggots are due to, at least in part, antibacterial action of three midgut lysozymes.

Keywords: lysozyme, maggot debridement therapy, *Lucilia sericata*, eradication of bacteria, midgut.

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Introduction

Lucilia sericata maggots are used for fast and effective treatment of non-healing wounds. Several clinical studies have provided compelling evidence that the application of sterile larvae of *L. sericata* to an infected non-healing wound results in the removal of necrotic tissue, disinfection, rapid elimination of infecting microorganisms and enhancement of the healing process (Parnés & Lagan, 2007; Zarchi & Jemec, 2012; Tian *et al.*, 2013). The mechanisms underlying the healing properties of maggots have been examined since the initiation of maggot debridement therapy (MDT) as a medical treatment option. It has been demonstrated that the mechanism of action results from both larval ingestion of wound bacteria, which appears to kill the bacteria as they pass through the larval digestive tract, and antimicrobial activity of larvae excretion/secretion (ES) products (Robinson & Norwood, 1934; Thomas *et al.*, 1999; Mumcuoglu *et al.*, 2001; Lerch *et al.*, 2003; Kerridge *et al.*, 2005; Jaklic *et al.*, 2008). Therefore, two groups of antibacterial factors are recognized in MDT: (1) compounds secreted/excreted from the salivary glands and gut into surrounding tissue, and (2) compounds secreted into the gut lumen, exhibiting rapid and local antibacterial activity against exogenous wound pathogens.

Most attention has been devoted to the identification and characterization of antibacterial compound(s) in ES products. Several studies have confirmed the antibacterial activity of *L. sericata* ES products against Gram-positive bacteria, but the activity against Gram-negative bacteria remained questionable (Thomas *et al.*, 1999; Kerridge *et al.*, 2005; Jaklic *et al.*, 2008). One of the well-described antibacterial compounds found in ES products is lucifensin (Cerovsky *et al.*, 2010), a novel larval defensin expressed in the salivary glands of all larval stages (Valachova *et al.*, 2013). Lucifensin is effective against *Staphylococcus carnosus*, *Streptococcus pyogenes* and *Streptococcus pneumoniae* (minimum inhibitory concentration, MIC 2 mg/l), as well as *Staphylococcus aures* (MIC 16 mg/l). The peptide did not show any antimicrobial activity towards Gram-negative bacteria (Andersen *et al.*, 2010). There are several other ES compounds possessing

antibacterial activity (Bexfield *et al*., 2004, 2008; Huberman *et al*., 2007); however, their chemical and structural characteristics are largely unknown.

Although it has been shown that a large number of bacteria are destroyed as they pass through the tubular gut of the maggot (Robinson & Norwood, 1934), no scientific data are available about the compounds secreted into the gut lumen that exhibit rapid and local antibacterial activity. Using green fluorescent protein (GFP)-producing *Escherichia coli*, it has been shown that a majority of *E. coli* is destroyed in the midgut during passage through the digestive tract (Mumcuoglu *et al*., 2001). However, the use of Gram-negative *E. coli* casts some doubt. Additionally, the mechanism of how bacteria are eradicated during passage through the gut has not been specified. Although based on a study with *Musca domestica*, it has been proposed that Cyclorrhapa larvae are able to kill bacteria in the middle region of the midgut through the combined action of low pH, lysozymes and cathepsin D-like proteinase (Espinoza-Fuentes & Terra, 1987; Lemos & Terra, 1991). However, no such study has been carried out using *L. sericata* larvae.

Using molecular approaches, several other putative antimicrobial proteins/peptides, including three novel lysozymes, have previously been found in the cDNA library of whole body maggots and predicted to be involved in MDT (Altincicek & Vilcinskas, 2009; Andersen *et al*., 2010). Lysozyme 1A (HM243538.1) and lysozyme 1B (HM243539.1) sequences were identified from the whole maggots cDNA library treated with transposon-assisted signal trapping, a technique developed for the identification of secreted proteins (Andersen *et al*., 2010). Lysozyme 2 (FG360533) was identified using the suppression subtractive hybridization method, which screens for genes that are differentially expressed in response to septic wounding (Altincicek & Vilcinskas, 2009). A significant induction of lysozyme 2 of about two to 10-fold has been observed in response to septic injury. Based on three-dimensional structure, it has been shown that *L. sericata* lysozyme 2 is similar to a digestive lysozyme from *M. domestica*. However, one exposed loop of *L. sericata* lysozyme 2 shows a significant difference in its structure, indicating possible variations in substrate recognition or in adaptation to different targets (Altincicek & Vilcinskas, 2009). Nevertheless, the exact tissue origin and the possible function of all three lysozymes in the MDT remain unknown.

Therefore, the aims of our study were to investigate the expression of previously identified *L. sericata* lysozymes (Altincicek & Vilcinskas, 2009; Andersen *et al*., 2010) and confirm their possible antimicrobial function during MDT. The eradication of bacteria within the alimentary tract of *L. sericata* maggots, in both Gram-positive and Gram-negative bacteria, was also investigated.

Results

Expression of lysozymes in the midgut of L. sericata

Using the specific cDNA probes, we detected strong expression of three previously identified lysozymes (Altincicek & Vilcinskas, 2009; Andersen *et al*., 2010) solely in the midgut of all larval stages. Expression of each lysozyme was tied to a specific part of the midgut.

Lysozyme 1A was expressed in the first half of the midgut of all larval stages. Expression was initiated at the base of the caeca and proceeded through the first half of the midgut. At the middle of the midgut, the expression gradually stopped (Fig. 1).

The expression of lysozyme 2 was initiated at the same part where the expression of lysozyme 1A terminated. Lysozyme 2 expression was tied to the shortest part of the midgut for all the proteins. The borders of the expression area were not sharp, and expression was initiated and also stopped gradually around the middle of the midgut (Fig. 2).

Lysozyme 1B expression was the weakest one. Only a few cells in the posterior part of the midgut expressed this protein during all stages of larval development. The expression area had no clearly defined borders, in contrast to those of the other two lysozymes. Expressing cells were evenly distributed along the posterior midgut (Fig. 3). The expression patterns of all three lysozymes were identical from the first to the third larval instar.

Recombinant expression and purification of lysozymes

The plasmids pET32-maLys1A/1B/2 containing the thioredoxin (Trx)-lysozyme fusion were transformed into *E. coli* Rosetta-gami 2 (DE3). Upon isopropyl-β-D-l-thiogalatopyranoside (IPTG) induction, lysozymes were expressed predominantly as part of the inclusion bodies.

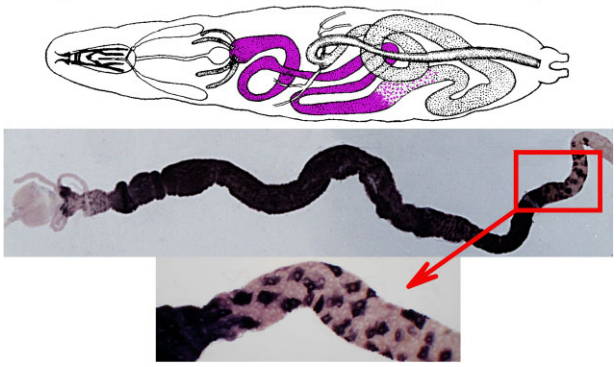

Figure 1. Expression of lysozyme 1A in the midgut of the third larval instar of *Lucilia sericata*. Lysozyme 1A expression was tied to the first half of the midgut. Expression was initiated at the base of the caeca and proceeded through the first half of the midgut. At the middle of the midgut, expression gradually stopped.

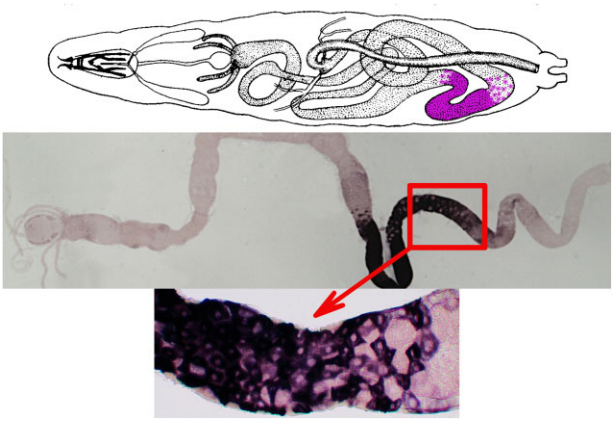

Figure 2. Expression of lysozyme 2 in the midgut of the third larval instar of *Lucilia sericata*. Lysozyme 2 expression was concentrated around the middle of the midgut. The borders of the expression area were not sharp, and expression was initiated and stopped gradually around the middle of the midgut.

According to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, there was an obvious recombinant protein band with a molecular weight of about 31 kDa, which was consistent with the predicted molecular weight of the recombinant lysozymes Trx-(His)6-maLys1A/1B/2 (data not shown). As the recombinant lysozymes contained a (His)6-tag, nickel-nitrilotriacetic acid (Ni-NTA) agarose resin was used for purification.

As each recombinant lysozyme was produced as an insoluble protein, it was necessary to refold the proteins by dialysing against 2l of 30 mM Tris, 20 mM L-arginine and pH 8.0. After refolding, recombinant lysozymes were pooled, dried and resuspended in 1 ml sterile distilled water, and tested for activity. The results of SDS-PAGE showed that the final refolded recombinant lysozymes each had a purity of 95% (Fig. 4). The final yield of the recombinant expression measured by Bradford assay was 1.20 mg/L on average (Table 1).

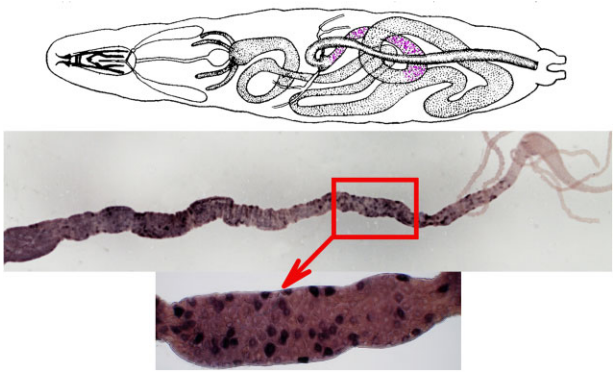

Figure 3. Expression of lysozyme 1B in the midgut of the third larval instar of *Lucilia sericata*. The expression of lysozyme 1B was localized in the posterior part of the midgut, but the area was not precisely defined. Only a few cells expressed lysozyme 1B and they were evenly distributed along the posterior midgut.

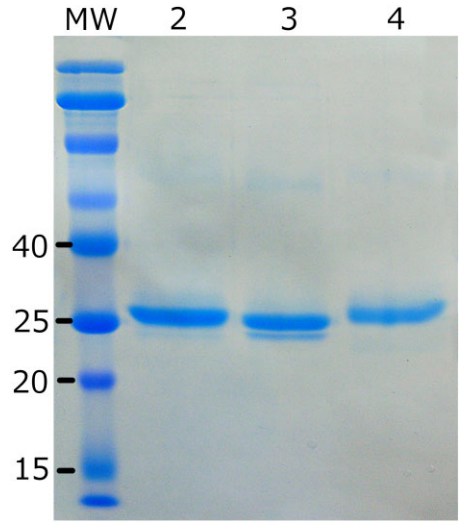

Figure 4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified and refolded recombinant thioredoxin (Trx)-lysozyme fusion proteins. According to analysis on 12% SDS-PAGE, the final refolded Trx-(His)6-maLys1A/1B/2 were produced to a purity of 95% each. Line 2, Trx-(His)6-maLys2; line 3, Trx-(His)6-maLys1A; line 4, Trx-(His)6-maLys1B. MW, molecular weight.

Antibacterial activity of recombinant lysozymes

The *in vitro* antibacterial activity of purified recombinant lysozymes was verified against both Gram-negative (*E. coli*) and Gram-positive (*Micrococcus luteus*) bacteria, using a MIC assay. All three recombinant lysozymes were active only against Gram-positive *M. luteus* with MIC values that ranged from 398 to 449 mg/l (Table 1). Recombinant lysozymes did not show any antibacterial activity against Gram-negative *E. coli* (data not shown).

Eradication of bacteria within the digestive tract of L. sericata

Using GFP-producing Gram-positive *Bacillus subtilis* and Gram-negative *E. coli*, we monitored the ability of bacteria to survive during passage through the intestine of *L. sericata* maggots. Vital bacterial cells strongly expressed GFP, which made them visible as green under fluorescent microscopy. In the lysed cells, the fluorescent

Table 1. Yield and minimum inhibitory concentration (MIC) values of the recombinant *Lucilia sericata* lysozymes

Name	Yield (mg/l)	*Micrococcus luteus* MIC (mg/l)
Lysozyme 1A	1.25	449
Lysozyme 1B	1.13	408
Lysozyme 2	1.33	398

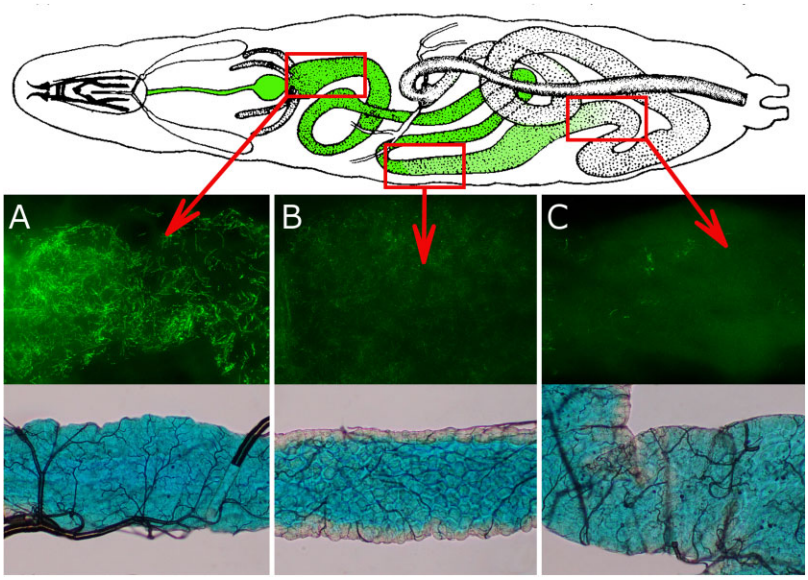

Figure 5. Eradication of Gram-positive *Bacillus subtilis* within the digestive tract of *Lucilia sericata*. *L. sericata* maggots were capable of eradicating the Gram-positive *B. subtilis* as they passed through their midgut. In the anterior part of the midgut, the fluorescent signal emitted by green fluorescent protein-producing *B. subtilis* cells was the most intense (A). In the middle midgut, it was clearly visible that the majority of *B. subtilis* cells were being lysed (B) and that complete cell lysis occurred beyond halfway along the midgut (C). All monitored parts of the guts were completely filled with food, which was visible because of the blue staining (top, schematic drawing; middle, fluorescent microscopy; bottom, light microscopy; column A, anterior midgut; column B, middle midgut; column C, posterior midgut).

signal was turned off; the GFP was released into the surroundings and was visible as a light green background. Concurrently, we used a vital blue dye as a control to visualize the food inside the gut of maggots under light microscopy.

Gram-positive *B. subtilis* were completely destroyed by the time they reached further than halfway along the midgut. In the anterior part of the midgut, the fluorescent signal from the bacterial cells was the most intense (Fig. 5A). In the middle midgut, it was clearly visible that the majority of bacteria cells were being lysed (Fig. 5B), with complete cell lysis occurring beyond halfway along the midgut (Fig. 5C).

Gram-negative *E. coli* were able to pass through the intestine of maggots almost unchanged. The strongest fluorescent signal was obtained from the anterior part of the midgut (Fig. 6A). It was a little bit weaker from the middle midgut (Fig. 6B) and was weakest from the hindgut. Nevertheless, a lot of intact cells were presented (Fig. 6C). During passage through the gut, the abundance of *E. coli* within the different parts of the gut changed slightly, but no cell lysis was observed. All monitored parts of the guts in both bacterial studies were completely filled with food, which was visible because of the blue staining of the food under light microscopy.

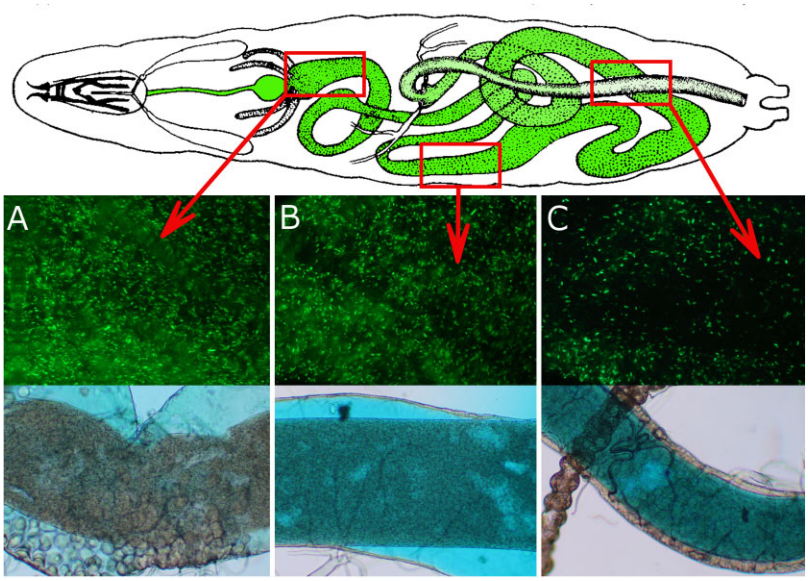

Figure 6. The ability of Gram-negative *Escherichia coli* to pass through the intestine of *Lucilia sericata* undamaged. Gram-negative *E. coli* was capable of passing through the intestine of *L. sericata* maggots undamaged. The majority of green fluorescent protein-producing *E. coli* was detected in the anterior part of the midgut (A). A small decrease in the amount of bacteria was visible in the middle midgut (B). As *E. coli* reached the posterior hindgut, their quantity was slightly reduced but cell lysis was not observed (C). All monitored parts of the guts were completely filled with food, which was visible because of the blue staining (top, schematic drawing; middle, fluorescent microscopy; bottom, light microscopy; column A, anterior midgut; column B, middle midgut; column C, posterior hindgut).

Discussion

In this study, we have shown that three previously identified *L. sericata* lysozymes (Altincicek & Vilcinskas, 2009; Andersen *et al*., 2010) are solely expressed in the midgut during all *L. sericata* larval stages, and that expression of these lysozymes takes place in a specific area within the midgut where the eradication of Gram-positive bacteria was observed. The expression of each lysozyme followed one after another along the gut. In addition, all recombinant lysozymes exhibited antibacterial activity *in vitro* against Gram-positive bacteria.

Several studies on different insect species have shown that genes encoding some digestive enzymes, metal transporters or antibacterial peptides are expressed in discrete parts of the gut (Abraham & Doane, 1978; Terra & Ferreira, 1994; Buchon *et al*., 2009; Shanbhag & Tripathi, 2009). The insect midgut has traditionally been divided into three regions based on the presence of acid-secreting cells in the adult middle midgut (Dimitriadis, 1991); the anterior, middle and posterior midgut. A recent study on *Drosophila melanogaster* led to the proposition of a subdivision of this organ into six major regions that are each divided into subregions. Each of the six major regions is delimited by boundaries that mark an anatomical constriction, a change in tissue histology and a site where gene expression patterns change at high frequency. Additionally, it has been shown that midgut compartmentalization is achieved through the interplay between pan-midgut and regionalized transcription factors, in concert with spatial activities of morphogens (Buchon *et al*., 2013). We assumed that the expression of the three lysozymes studied here in specific regions of the *L. sericata* midgut is under the control of specific transcription factors.

In most insects, lysozymes are a prominent component of their defence and it is clear that they are present in the haemolymph at increased levels after infection (Hultmark, 1996). An exception is in cyclorrhaphous Diptera (dung-feeding larvae such as *M. domestica* or fruit-feeding larvae such as *D. melanogaster*), in which lysozymes are not expressed at significant levels in the fat body or haemocytes, and the genes are not induced in parallel with cercopin, diptericin and other bactericidal factors, but are produced in the midgut (Lemos & Terra, 1991; Kylsten *et al*., 1992; Daffre *et al*., 1994). Although lysozymes are not expressed in the fat body or haemocytes in flies, a constitutive level of lysozyme activity is normally present in the haemolymph. Surprisingly, the same lysozymes are found in the haemolymph as found in the gut, and it appears that the midgut tissue can export lysozymes in two directions, to the gut lumen and to the haemocoel (Hultmark, 1996). A significant induction of *L. sericata* lysozyme 2 of about two to 10-fold has been observed in response to septic injury (Altincicek & Vilcinskas, 2009). Surprisingly, we did not detect any change in the localization of lysozyme expression upon septic injury with lipopolysaccharide solution. All lysozymes were expressed only in the midgut (data not shown). This could mean that the previously detected increase in the production of lysozyme 2 took place only within the gut, from where lysozyme 2 could also have been exported to the haemocoel.

It has been predicted that *L. sericata* lysozymes could be involved in MDT in a similar way to other antibacterial factors (Altincicek & Vilcinskas, 2009; Andersen *et al*., 2010). In order to clarify their functions, we prepared each lysozyme in its recombinant form and showed that all three lysozymes were comparatively effective against Gram-positive *M. luteus*. Lysozymes are mostly known for their defensive role against bacteria but in several animals, lysozymes have been recruited for digestive purposes (Hultmark, 1996). Like other insects (and like most other animals), the early ancestors of modern dipterans were probably equipped with a single typical c-type lysozyme, expressed in the blood and used in defence against bacteria. Following the development of an acidic midgut and the recruitment of lysozymes for digestive purposes, which occurred after the divergence of the Cyclorrhapha from the Nematocera (Lemos & Terra, 1991), the number of fly lysozyme genes was expanded (Beverley & Wilson, 1984). Many cyclorhaphan flies, including *L. sericata*, live on decomposing matter. Therefore, an efficient system to digest microorganisms was an important prerequisite for occupying this niche successfully. Two digestive lysozymes were purified from the *M. domestica* midgut (Lemos *et al*., 1993; Ito *et al*., 1995). MdL1 and MdL2, expressed as recombinant proteins in *Pichia pastoris*, displayed lytic activities upon *Micrococcus lysodeikticus* (Cancado *et al*., 2008). In *D. melanogaster*, at least seven different lysozyme genes are expressed in different parts of the digestive tract. The major lysozymes in the *D. melanogaster* gut have acidic isoelectric points and/or pH optima (Regel *et al*., 1998). This is highly reminiscent of the situation in ruminants, in which the lysozymes have also been recruited for the digestion of symbiotic bacteria in the stomach (Irwin, 1996).

It has been proposed that Cyclorrhapa larvae are able to kill bacteria in the middle region of the midgut through the combined action of low pH, lysozymes and cathepsin D-like proteinase (Espinoza-Fuentes & Terra, 1987; Lemos & Terra, 1991). The production of lysozymes in the midgut seems to be crucial for this, but only the Gram-positive bacteria are destroyed as they passed through the midgut. Our results also show that lysozymes 1A and 1B are expressed in the near-neutral (anterior) and alkaline (posterior) part of the midgut, respectively, whereas

Table 2. Primers used to synthesize the cDNA fragments for *Lucilia sericata* lysozymes probes

Name/GenBank	Sense primer	Antisense primer	Final amplicon (bp)
Lysozyme 1A HM243538.1	5′-ACATTCACTCGCTGCTCCTT-3′	5′-GCTACCGCTGCAGTATTTCC-3′	339
Lysozyme 1B HM243539.1	5′-TTCATCAATTTCTTCGTTATAGCC-3′	5′-CCCAAGTAGTCCAGGCAGAC-3′	379
Lysozyme 2 FG360533	5′-ATTCGTCATTTTGGCTGCTT-3′	5′-AGCACCAGATGGAGGAGAAC-3′	250

lysozyme 2 is produced strictly in the middle midgut, which is acidic. The comparison of the amino acid sequences of all three lysozymes indicated that lysozyme 2 is slightly different, probably because of its adaptation to the acidic part of the middle midgut. Lysozyme 2 is in this way similar to the digestive lysozymes found in *M. domestica* (Espinoza-Fuentes & Terra, 1987; Lemos & Terra, 1991) and four lysozymes in *D. melanogaster* (Daffre *et al.*, 1994), which are also expressed in the acidic part of the midgut.

It has been assumed that one of the mechanisms underlying the antibacterial action of maggots during MDT may result from larval ingestion of wound bacteria (Robinson & Norwood, 1934; Mumcuoglu *et al.*, 2001; Lerch *et al.*, 2003). Using GFP-producing Gram-positive *B. subtilis* and Gram-negative *E. coli*, we showed that larvae of *L. sericata* are capable of eradicating only Gram-positive bacteria within their midguts. Gram-negative bacteria could pass through the larval intestinal system undamaged. Our results are consistent with previous findings that several Gram-negative bacteria species, including *Proteus mirabilis*, can colonize maggots' alimentary tracts (Jaklic *et al.*, 2008). This might be because of either bacterial adaptation or their symbiotic relationships as members of the *L. sericata* gut flora. However, it has been proved that MDT can be successfully used to treat wounds associated with Gram-negative bacteria (Kerridge *et al.*, 2005). One possible explanation is that the eradication of such pathogens during MDT is associated with the activity of skin cells (particularly keratinocytes) that secrete antimicrobial peptides into the environment when activated. Several effects of larval ES on molecules and cells of the immune system, and cells involved in wound repair, such as fibroblasts or keratinocytes, have been proposed (Cazander *et al.*, 2013).

In conclusion, we have shown that three previously identified *L. sericata* lysozymes (Altincicek & Vilcinskas, 2009; Andersen *et al.*, 2010) are expressed in specific sections of the midgut during larval development. Prepared recombinant lysozymes displayed comparable antimicrobial activity against *M. luteus* but were ineffective against *E. coli*. Larvae of *L. sericata* are capable of eradicating Gram-positive bacteria within the same specific section of the midgut where lysozymes are produced. Taken together, *L. sericata* lysozymes are involved in the eradication of Gram-positive bacteria during MDT.

Experimental procedures

Rearing of L. sericata *larvae*

Colonies of the green bottle fly *L. sericata* were maintained at the Institute of Zoology, Slovak Academy of Sciences (SAS), under constant conditions. Imagos were exposed to 12 h light/dark photocycles at 25 ± 1 °C and a relative humidity of 40–50%. The larvae were fed on ground beef liver mixed with bran.

Synthesis of the cDNA fragments encoding lysozymes

The sequence information obtained from GenBank for the three previously identified *L. sericata* lysozymes (Altincicek & Vilcinskas, 2009; Andersen *et al.*, 2010) was used for the expression study. Primers for the synthesis of cDNA fragments, which served as templates to synthesize the digoxigenin (Dig)-labelled single-stranded antisense DNA probes, were designed based on the cDNA sequence (Table 2).

In situ *hybridization*

In situ hybridization was used to detect and localize the expression of lysozymes in different tissues of maggots during larval development. We used all three larval instars during the feeding stages because these stages are used during MDT. The amplicons of three lysozymes were re-amplified by asymmetric PCR, using the PCR Dig Probe synthesis kit (Roche Applied Science, Penzberg, Germany) and antisense primers to synthesize the Dig-labelled single-stranded antisense DNA probes. The Dig-labelled probes were purified using a PCR purification kit (Promega, Madison, WI, USA) and stored at −20 °C. The salivary glands, gut, central nervous system and fat body were dissected under phosphate-buffered saline (PBS, pH 7.2) from the larvae of different stages, fixed in 4% paraformaldehyde at 4 °C overnight and subjected to the whole-mount *in situ* hybridization procedure as described previously (Kim *et al.*, 2006). The tissues from three to five individuals were studied for each hybridization probe and the negative controls were performed using the specific sense probes. Colour development was controlled under a binocular microscope and stained tissues were mounted in glycerol. The intensity of expression was determined by the appearance of the tissues under a microscope (Motic, type 107M, Motic Deutschland GmbH, Wetzlar, Germany).

Strains, vectors, enzymes and reagents

Escherichia coli strain JM109 was used as the host for gene cloning and DNA manipulation. *E coli* Rosetta-gami 2 (DE3) was purchased from Novagen (Madison, WI, USA) and used as the host for expression of the heterologous protein. Bacteria were grown in Luria–Bertani (LB) medium or LB agar plates with

Table 3. Primers used to synthesize the *Lucilia sericata* lysozyme inserts for construction of expression plasmids

Insert	Sense primer	Antisense primer
maLyz1A	5′-GACGACGACGACAAGAAGACATTCACCCGC-3′ 5′-CCGGTACCGACGACGACGACAAGAAGAC-3′	5′-CCGCTCGAGTTAGAAACAATCATTAATGCTGGG-3′
maLyz1B	5′-GACGACGACGACAAGAAGACTTTTACACGC-3′ 5′-CCGGTACCGACGACGACGACAAGAAGAC-3′	5′-CCGCTCGAGTTAAAAACATTCATCAATACTTGG-3′
maLyz2	5′-GACGACGACGACAAGAAAGTTTATACACGTTGCTC-3′ 5′-CCGGTACCGACGACGACGACAAGAAAGTTTATAC-3′	5′-CCGCTCGAGTTAAAAACAATCGTCAATGCTGG-3′

Underlined sequences indicate the restriction enzyme *Kpn*I-recognized site in sense primers and *Xho*I in antisense primers.

appropriate antibiotics at 37 °C (ampicillin 25 mg/l in H_2O, chloramphenicol 34 mg/ml in ethanol and tetracycline 5 mg/ml in ethanol). *E. coli* cells were transformed using the standard heat-shock method. Plasmid pET32a(+) (Novagen) was chosen for the construction and expression of fusion proteins. Isis DNA Polymerase was purchased from MP Biomedicals (Santa Ana, CA, USA), T4 DNA Ligase and restriction enzymes from New England BioLabs (Beverly, MA, USA) and other chemicals from Sigma (St Louis, MO, USA) or Promega (Madison, WI, USA).

Construction of the expression plasmids

We used the pET32a (+) plasmid, which provides Trx as a fusion partner to increase the solubility of the targprotein in the *E. coli* cytoplasm. All expression plasmids for each lysozyme were constructed in the same way. We synthesized the cDNA fragment of the mature lysozyme with a *Kpn*I site and enterokinase (EK) recognition sequence on the N-terminal and a *Xho*I site on the C-terminal. Primers for the synthesis of cDNA fragments, which served as inserts for construction of expression plasmids, were designed based on the cDNA sequence and used in nested PCR (Table 3). Synthesized cDNA fragments were purified, digested with *Kpn*I and *Xho*I and then ligated into the pET32a(+) vector, which was also digested with these two restriction enzymes, to construct the expression plasmids, pET32-maLys1A/1B/2, respectively. The resulting plasmids were transformed into JM 109 *E. coli* and verified by DNA sequence analysis (GATC Biotech, Constance, Germany). The correct plasmids encoded a translational fusion peptide containing an N-terminal Trx part, followed by a (His)6-tag, an EK cleavage site and the mature peptide sequence of lysozyme 1A/1B/2, respectively (hereinafter designated as recombinant lysozyme).

Expression of the recombinant lysozymes

Escherichia coli Rosetta-gami host strains can enhance disulphide bond formation. For this reason, the confirmed expression vectors were transformed into *E. coli* Rosetta-gami 2 (DE3) to generate the *E. coli*/pET32-maLys. To express the recombinant lysozymes, the desired *E. coli*/pET32-maLys clones were selected and inoculated into 3 ml LB medium supplemented with ampicillin, chloramphenicol and tetracycline culturing at 37 °C overnight with shaking at 250 rpm. The overnight cultures were diluted 100-fold into fresh LB medium (total volume of expression for each recombinant lysozyme was 1200 ml) with antibiotics. When the cell cultures were grown until reached the absorbance (measured at 600 nm) at about 0.5 (mid-log phase of growth), the expression of recombinant lysozymes was induced by the addition of IPTG to a final concentration of 0.8 mM. After additional overnight culture following the induction of IPTG at room temperature (RT), the harvested cells were immediately centrifuged at 5000 *g* for 10 min.

Purification of the recombinant lysozymes

Expressed recombinant lysozymes from the harvested cells were extracted using B-Per Reagent (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Insoluble inclusion bodies were solubilized in denaturing buffer (6 M urea, 0.1 M NaCl) and the recombinant lysozymes containing (His)6-tag were purified using Ni-NTA agarose affinity chromatography (Sigma-Aldrich, St Louis, MO, USA). The recombinant lysozymes bound to the resin were washed using washing buffer (6 M urea, 0.1 M NaCl, 30 mM imidazol), and then eluted with elution buffer (6 M urea, 0.1 M NaCl, 0.25 M imidazol). After elution, recombinant lysozymes were dialysed against 30 mM Tris, 20 mM L-arginine, pH 8.0 at 4 °C overnight, lyophilized and re-suspended in 1 ml sterile distilled water. The purified recombinant lysozymes were analysed by 12% SDS-PAGE and their concentrations were measured by a Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA, USA).

Antibacterial activity assay

The antibacterial activity of purified recombinant lysozymes was determined against laboratory strain *M. luteus* ATCC 272 at the MIC in a microtitre plate. One bacterial colony from a 5% blood agar plate incubated overnight was suspended in PBS buffer at pH 7.2 and the turbidity of suspension was adjusted to 10^8 colony forming units (CFU)/ml and diluted in Mueller-Hinton broth (MHB) to a final concentration of 10^6 CFU/ml. Ten-microlitre aliquots of suspension were inoculated into each well of sterile 96-well polystyrene plates (Sarstedt, Nümbrecht, Germany). The bacterial suspension in MHB was incubated with different volumes (10, 20, 30 μl) of stock solution of recombinant lysozymes in a total volume of 100 μl per well. After 18 h of incubation at 37 °C, bacterial growth inhibition was determined by monitoring the absorbance at 450 nm. The MIC was defined as the lowest concentration of lysozymes that inhibited bacterial growth.

In vivo *monitoring of bacteria in the intestines of maggots*

To image the bacteria in the intestines of the *L. sericata* maggots, we used GFP-producing bacteria: Gram-negative *E. coli* JM 109 transformed with *pGreen* plasmid carrying GFP (Caroline Chemistry, Burlington, NC, USA) and GFP-producing Gram-positive *B. subtilis* IB 1242 (Jamroskovic *et al.*, 2012) (obtained as a kind gift from the Institute of Molecular Biology, SAS). One bacterial colony of overnight agar plate culture of GFP-expressing bacteria

strains was suspended in PBS, and the turbidity of suspension was adjusted to 10^8 CFU/ml. A 100-µl aliquot of suspension was inoculated to 10 ml of melted LB containing 0.7% (w/v) agar pre-heated at 48 °C with a blue vital dye (0.1 mg/ml), and poured into 90-mm Petri dishes. After solidification, plates were incubated at 37 °C overnight to allow bacterial growth. Sterile maggots in the early third instar were placed on the plates and incubated for 2 h. Infected maggots were then washed in sterile water and dissected in a drop of PBS under a stereomicroscope. The apical and posterior ends of the maggot were removed and the body contents squeezed out of the cuticula. The alimentary tract was uncoiled by removing the tracheae, the fat bodies and the Malpighian tubules. The gut was fixed in 4% paraformaldehyde at RT for 1 h, washed three times with PBS containing 0.2% Tween-20 and moulted in glycerol. The presence of bacteria in the different parts of the intestinal tract was monitored using a Motic microscope (BA 410) with a single band pass filter for fluorescent microscopy, and photographed with a Canon digital camera (EOS 1100D, Tokyo, Japan).

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