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Antimicrobial Peptides Expressed in Medicinal Maggots of the Blow Fly *Lucilia sericata* Show Combinatorial Activity against Bacteria

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The larvae of the common green bottle fly (*Lucilia sericata*) produce antibacterial secretions that have a therapeutic effect on chronic and nonhealing wounds. Recent developments in insect biotechnology have made it possible to use these larvae as a source of novel anti-infectives. Here, we report the application of next-generation RNA sequencing (RNA-Seq) to characterize the transcriptomes of the larval glands, crop, and gut, which contribute to the synthesis of antimicrobial peptides (AMPs) and proteins secreted into wounds. Our data confirm that *L. sericata* larvae have adapted in order to colonize microbially contaminated habitats, such as carrion and necrotic wounds, and are protected against infection by a diverse spectrum of AMPs. *L. sericata* AMPs include not only lucifensin and lucimycin but also novel attacins, cecropins, diptericins, proline-rich peptides, and sarcotoxins. We identified 47 genes encoding putative AMPs and produced 23 as synthetic analogs, among which some displayed activities against a broad spectrum of microbial pathogens, including *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Enterococcus faecalis*. Against *Escherichia coli* (Gram negative) and *Micrococcus luteus* (Gram positive), we found mostly additive effects but also synergistic activity when selected AMPs were tested in combination. The AMPs that are easy to synthesize are currently being produced in bulk to allow their evaluation as novel anti-infectives that can be formulated in hydrogels to produce therapeutic wound dressings and adhesive bandages.

The larvae of the common green bottle fly *Lucilia sericata*, a member of the blow fly family (Calliphoridae), are known as medical maggots or wound maggots, because they have been used to treat wounds as part of traditional medicine (1). This approach, commonly termed maggot therapy, is an approved and prospering alternative for the treatment of chronic and nonhealing wounds, e.g., those associated with diabetic ulcers (2–4). The therapeutic effects include the removal of necrotic tissue (debridement), the acceleration of wound healing, and wound disinfection (5, 6). The wound disinfection property has been attributed to antimicrobial components in the larval secretions, including small molecules with antibacterial activity (7, 8) and antimicrobial peptides (AMPs), such as the defensin-like lucifensin (9) and a recently reported AMP with potent antifungal activity, which accordingly was named lucimycin (10).

The ability of *L. sericata* larvae to prosper on carrion and necrotic wounds suggests that they are adapted to colonize contaminated environments, e.g., by expressing a diverse spectrum of AMPs to protect them against the microbes they encounter (11). This theory is supported by the unique ability of rat-tailed maggots (larvae of the drone fly *Eristalis tenax*) to survive in highly contaminated aquatic habitats, such as liquid manure storage pits and cesspools, which also reflects their production of multiple diverse AMPs (12). Similarly, we found that the burying beetle *Nicrophorus vespilloides*, which lives and reproduces on cadavers, also produces a wide spectrum of AMPs (13). The synthesis of diverse AMPs also provides the potential for beneficial combinatorial interactions, such as additive antimicrobial effects, synergy (greater-than-additive effects), or potentiation (one AMP enabling or enhancing the activity of others). The diversification of genes encoding AMPs by duplication and sequence divergence is often replicated at the functional level, thus providing protection against a broader spectrum of microbes (11, 14).

To test the hypothesis that *L. sericata* is protected from infection by its AMP repertoire, we injected larvae with bacteria and cataloged the transcriptomes by high-throughput RNA sequencing (RNA-Seq) to identify the inducible immunity-related genes. In order to separate AMPs that are secreted into wounds from those with other functions, we compared the transcriptomes of whole larvae, dissected salivary glands, crops, and gut samples, because antimicrobial activity has been detected in oral secretions and is also regurgitated from the gut (15, 16). We identified an impressive spectrum of genes encoding 47 putative AMPs, many of which were found to be differentially expressed in the relevant tissues. We produced 23 of the novel *L. sericata* AMPs as synthetic peptides to test their activities, alone or in combination, against bacteria.

MATERIALS AND METHODS

Biological samples. *L. sericata* maggots were obtained from BioMonde GmbH (Barsbüttel, Germany) and maintained under sterile conditions on blood agar plates (Columbia Agar with Sheep Blood Plus; Oxoid,

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United Kingdom) at 28°C in the dark. A prescreening experiment was used to identify the optimal bacterial species and the inoculation time for maximum immune induction. For each experimental group, 10 larvae (~5 mm in length) reared on blood agar plates for 48 h were placed in petri dishes on ice to reduce their mobility and facilitate injection. The bacteria were introduced ventrolaterally by pricking the abdomen using a dissecting needle dipped in an aqueous solution of individual or mixed bacterial cultures (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Micrococcus luteus*), or in phosphate-buffered saline (PBS). Hemolymph samples were taken from five larvae in each treatment group 8 and 24 h after injection and tested for antimicrobial activity in bacterial plate growth inhibition assays. Based on these initial assays, we used a mixture of *P. aeruginosa* (strain DSM 50071) and *S. aureus* (strain DSM 20044) for the subsequent immune induction experiments. In this new series of experiments, the eight infected larvae and 15 controls were shock frozen in liquid nitrogen 8 h after injection and stored at −80°C. Pools of infected or control larvae (first and third instar) were used to prepare RNA samples for the 454-FLX-based sequencing of normalized RNA and the Illumina-based sequencing of non-normalized RNA. The infected and control third instar larvae were also dissected to separate the salivary glands, crops, and guts from the rest of the body to prepare the RNA samples for Illumina RNA-Seq analysis.

RNA isolation. Total RNA was extracted from each of the pooled larval samples and tissues described above using the innuPREP RNA mini isolation kit (Analytik Jena, Jena, Germany), followed by additional RNA purification, quantification, and quality control assays, as previously described (17). For the isolation of mRNA from whole larvae, an additional DNase treatment (Turbo DNase; Ambion Life Technologies, Darmstadt, Germany) was applied prior to the second purification step, and the DNase was removed before further RNA purification using the RNeasy MinElute cleanup kit (Qiagen, Venlo, the Netherlands). The pure RNA was eluted in 20 μl of RNA storage solution (Ambion Life Technologies). Poly(A)$^+$ mRNA was isolated from total RNA using the Ambion MicroPoly(A)Purist kit, according to the manufacturer's instructions (Life Technologies), and the integrity and quantity were verified using an Agilent 2100 bioanalyzer and RNA Nano chips (Agilent Technologies, Santa Clara, CA, USA).

Roche 454 sequencing of normalized larval RNA. To maximize sequence diversity, we pooled RNA extracted from different larval stages and from control and immunochallenged larvae and normalized the RNA pool. For 454 sequencing, a full-length, enriched, and normalized cDNA library was generated from the poly(A)$^+$ mRNA using a combination of the SMART cDNA library construction kit (Clontech, TaKaRa, Saint-Germain-en-Laye, France) and the Trimmer Direct cDNA normalization kit (Evrogen, BioCat, Heidelberg, Germany), as previously described (18). The optimization of the complete cDNA normalization procedure was essentially carried out as described by Vogel and Wheat (19). Normalized cDNA was purified on DNA Clean & Concentrator columns (Zymo Research, Freiburg, Germany) for direct sequencing. The cDNA library from the normalization procedure was sequenced on the Roche 454 FLX platform using Titanium chemistry at GATC Biotech (Constance, Germany). A full microtiter plate run resulted in a total of 1,089,962 reads, with an average read length of 346 bp after tag removal and quality trimming.

Illumina sequencing, transcriptome assembly, and annotation. Larval and tissue-specific transcriptome sequencing of five different mRNA pools was carried out on an Illumina HiSeq 2000 genome analyzer platform using either single-end (1 × 100 bp) (whole larva) or paired-end (2 × 100 bp) (larval tissue) read technology with RNA fragmented to an average of 150 nucleotides. Sequencing was carried out by Eurofins MWG Operon (http://www.eurofinsgenomics.eu) and resulted in a total of 28, 30, 26, 32, and 104 million reads for the rest of the body, gut, crop, glands, and whole-larva samples, respectively. Quality control measures, including the filtering of high-quality reads based on the score given in fastq files, the removal of reads containing primer/adapter sequences, and the trimming of read length, were carried out using CLC Genomics Workbench version 6.5. The *de novo* transcriptome assembly was carried out using the same software by comparing an assembly with standard settings and two additional CLC-based assemblies with different parameters and then selecting the presumed optimal consensus transcriptome, as described previously (20). Any conflicts among the individual bases were resolved by voting for the base with highest frequency. Contigs <200 bp were removed from the final analysis. The resulting final *de novo* reference transcriptome assembly (backbone) contained 67,193 contigs >200 bp, with an N_{50} contig size of 1,097 bp and a maximum contig length of 27,637 bp. The transcriptome was annotated using BLAST, Gene Ontology, and InterProScan searches with Blast2GO Pro version 2.6.1 (21). For BLASTx searches against the nonredundant NCBI protein database (NR database), up to 20 best NR hits per transcript were retained, with an E value cutoff of $\leq 10^{-1}$ and a minimum match length of 15 amino acids to obtain the best homolog for the predicted short polypeptides. Annex (22) was used to optimize the Gene Ontology (GO) term identification further by crossing the three GO categories (biological process, molecular function, and cellular component) to search for name similarities, GO term relationships, and enzyme relationships within metabolic pathways (Kyoto Encyclopedia of Genes and Genomes) (23). To identify candidate genes in the *L. sericata* transcriptome assembly, we established a reference set of known or predicted insect-derived AMPs using published sequences and by searching our in-house database and public databases (NCBI).

Digital gene expression analysis. Digital gene expression analysis was carried out using the QSeq software (DNAStar, Inc.) to remap the Illumina reads from all five samples onto the reference backbone and then counting the sequences to estimate expression levels, using previously described parameters for read mapping and normalization (24). Biases in the sequence data sets and different transcript sizes were corrected using the RPKM algorithm (reads per kilobase of transcript per one million mapped reads) to obtain correct estimates for the relative expression levels. To control for the effect of global normalization using the RPKM method, we also analyzed a number of highly conserved housekeeping genes that are used as control genes for quantitative PCR. These included several genes encoding ribosomal proteins (rpl3, rpl4, rpl13, rpl15, rps2, rps8, rps12, rps15a, rps18, and rps24), elongation factor 1-alpha, and eukaryotic translation initiation factors 4 and 5. The corresponding contigs were inspected for the overall expression levels across the samples and treatments and were found to display expression-level differences (based on RPKM values) of <1.3-fold between samples, indicating that they were not differentially expressed and validating them as housekeeping genes.

Synthetic AMPs. We synthesized four proline-rich peptides, two stomoxyns, six defensins, six cecropins, and four putative cecropin-like peptides that were identified in the *L. sericata* transcriptome (Table 1). The peptides were produced by solid-phase synthesis and purified by Coring System Diagnostix (Gernsheim, Germany) and GenScript (Piscataway, NJ, USA). The integrity of the peptides was confirmed by liquid chromatography-mass spectrometry (LC-MS). The peptides were dissolved in dimethyl sulfoxide (DMSO) (*N*,*N*-dimethylformamide [DMF] for defensins) to a concentration of 10 mM and were then diluted in sterile distilled water to a concentration of 400 μM.

Bacterial cultures and AMP activity assays. *E. coli* strain D31 was cultivated in LB medium (Roth, Karlsruhe, Germany) and *M. luteus* strain DSM 20030 in tryptic soy broth (Roth) at 37°C. The bacterial strains were cultivated overnight and were subcultured onto fresh medium and grown for 4 h before testing.

We used a photometric assay to determine the growth of bacteria in liquid cultures. Serial 2-fold dilutions of AMPs (200 μM to 0.8 μM in the appropriate bacterial growth medium) were transferred to 384-well microtiter plates in 10-μl aliquots. The *E. coli* and *M. luteus* cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.0004 and 0.001, respectively, and 10-μl aliquots were added to each well. As a control, the appropriate solvent was added to the bacterial suspensions instead. The plates were incubated in an Eon microplate spectrophotometer (BioTek, Seattle, WA, USA) for 16 h at 37°C, and the OD_{600} was measured at

TABLE 1 Properties of 23 synthetic antimicrobial peptides identified in the transcriptome of *L. sericata*

Peptide	Sequence	pI	Molecular mass (g/mol)
Putative cecropin-like			
LSer-PCecL2	HHHHRFGKIGHELHKGVKKVEKVTHDVNKVTSGVKKVASSIEKAKNV	10.2	5,260
LSer-PCecL3	HHHFGRIGHELHKGVKKVEKVTSDVNKVTNGVKQVANGIAKAKTVIEAGSIAGAVAAAAA	10.0	6,122
LSer-PCecL4	HHLFGKVGREIERSAHKVGHKLEHVRHEVSKTAKKVDKVVGHIKTAKKVVAAAGAIAGVVAAA	10.4	6,664
LSer-PCecL5	HHHLFGHVGHEVERSLHKVGHKLEHACHEVHKTAKKVQK	9.4	4,546
Cecropins			
LSer-Cec1	GWLKKIGKKIERVGQHTRDATIQTIGVAQQAANVAATLKG	11.4	4,319
LSer-Cec2	GWLRDFGKRIERVGQHTRDATIQAIGVAQQAANVAATVRG	10.6	4,258
LSer-Cec3	GWLKKIGKKIERVGQHTRDATIQVLGVAQQAANVAATARG	11.1	4,242
LSer-Cec4	GWLKKIGKKIERVGQHTRDASIQAIGIAQQAANVAATARG	11.1	4,127
LSer-Cec5	GLVKKIGKKIERVGQHTRDASIQAIGIAQQAANVAATARG	10.6	4,262
LSer-Cec6	GWLKKFGKKIERVGQHTRDATIQAIGVAQQAANVAATLKG	11.1	4,325
Proline-rich			
LSer-PRP1	EWRPHGSNGGSSLRPGRPQTLPPQRPIQPDFNGPRQRF	12.0	4,322
LSer-PRP2	EWRPHGSIGGSGLRPGRPQTLPPQRPRRPDFNGPRHRF	12.2	4,371
LSer-PRP3	SPFVDRPRRPIQHNGPKPRIITNPPFNPNARPAW	12.2	3,945
LSer-PRP4	SWIKKDKFPSSTGPYNPNPPPPRF	10.0	2,758
Stomoxyns			
LSerStomox1	AGFRKRFNKLVKKVKHTIKETANVSKDVAIVAGSGVAVGAAMG	10.7	4,455
LSerStomox2	GFRKRFNKLVKKVKHTIKETANVSKDVAIVAGSGVAVGAAMG	10.7	4,384
Defensins			
LSer-Def1	ATCDLLSATGFSGTACAAHCLLIGHRGGYCNTKSVCVCRD	7.8	4,077
LSer-Def2	ATCDLLSGTGVKHSACAAHCLLRGNRGGYCNGRAICVCRN	9.0	4,123
LSer-Def3	ATCDLLSGTGANHSACAAHCLLRGNRGGYCNSKAVCVCRN	8.7	4,069
LSer-Def4	LTCNIDRSFCLAHCLLRGYKRGFCTVKKICVCRH	9.4	3,959
LSer-Def6	GTCSFSSALCVVHCRVRGYPDGYCSRKGICTCRR	9.2	3,742
LSer-Def7	FTCNSYACKAHCILQGHKSGSCARINLCKCQR	9.2	3,545
Antifungal			
LSer-AFP	QHGYGAGGHGQQGYGSQHSSHAPQGGHVVREQGFSGHVHEQQAGHHHEAGHHEQAGHHEQ SGQQVHGQGHGYKSHGY	6.6	8,200

20-min intervals. The MIC was determined as the lowest peptide concentration causing the complete inhibition of bacterial growth.

Activity testing against further microbial pathogens was performed at the Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute (HKI), Jena, Germany. Antimicrobial activity was determined by agar diffusion tests, as described previously (25). Shortly, a 100-μl suspension of the test organism (listed in Table S1 in the supplemental material) with a density of 0.5 McFarland standard was inoculated into 32 ml of melted agar medium and poured into petri dishes. Holes of 9 mm in diameter were cut in the agar and filled with 50 μl of a selected compound solution. The inhibition zones were read after overnight incubation.

Sensitivity of bacteria to combinations of AMPs. Two peptide solutions were diluted to initial concentrations of eight times the predetermined MICs in a 384-well microtiter plate. The solutions were combined in ratios of 1:5, 1:3, 3:1, and 5:1. We then added 10 μl of the single and combined AMP solutions to the 384-well plates, and 2-fold serial dilutions were prepared so that six rows of the two individual AMPs and the four combinations could be tested in parallel. The AMP activity assay was carried out as described above, and the MIC was determined for each combination. Isobolograms were constructed by plotting the fractional inhibitory concentration (FIC) of peptide A against peptide B for each of the peptide ratios. The inhibitory concentration of the peptides in combination was expressed as the fractions of the inhibitory concentration of the peptides alone, normalized to unity: FIC(A) = MIC of peptide A in combination/MIC of peptide A alone. A concave curve indicated synergy, a straight line indicated additive activity, and a convex curve indicated antagonism. In addition, the sum of the fractional inhibitory concentration (sumFIC) was calculated using the following equation: sumFIC = FIC(A) + FIC(B). The sumFIC results were interpreted as follows: a sumFIC of $\leq$0.5 indicates synergy, a sumFIC of $>$0.5 and $\leq$1 indicates additive effects, and a sumFIC of $<$2 indicates antagonism. The sumFIC values were calculated according to the peptide combination representing the point closest to the middle of the isobologram.

Nucleotide sequence accession number. We have deposited the short-read (Illumina HiSeq 2000) data under the accession number PRJEB7567 (EBI Short Read Archive [SRA]). The complete study can be accessed directly at http://www.ebi.ac.uk/ena/data/view/PRJEB7567.

RESULTS

Identification and organ-specific expression of AMPs. Our comprehensive transcriptomic data set from whole larvae and dissected organs (salivary glands, crop, and gut) was screened extensively for cDNAs encoding AMPs using both our in-house database of insect-derived AMPs and public databases. This resulted in the identification of 47 putative AMPs belonging to diverse families and functional classes. A quantitative RNA-Seq analysis of the transcripts revealed that most of the AMPs were differentially expressed, and this was visualized by preparing a

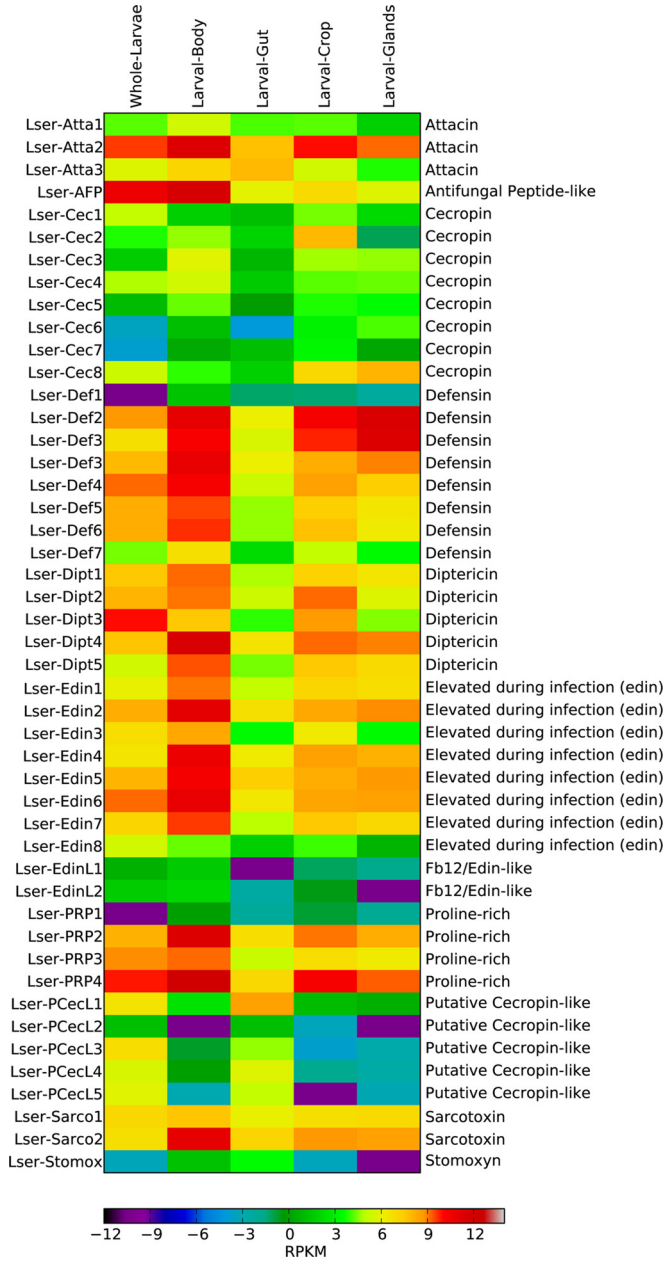

FIG 1 Heat map showing relative expression levels of *L. sericata* antimicrobial peptides. The abbreviations of the individual AMPs are depicted on the left, while AMP classes are depicted on the right. Shown are $\log_2$-transformed RPKM values (blue resembles lower-expressed genes, while red represents highly expressed genes).

heat map to compare the normalized mapped read (RPKM) values of each AMP across all samples (Fig. 1).

We identified three putative members of the attacin family, eight putative members of the cecropin family, five additional cecropin-like peptides, a stomoxyn (structurally related to cecropins), and two sarcotoxins. Despite the structural similarities of these AMPs, we classified them according to the established nomenclature in the literature. In addition, we found eight defensin-like peptides, five putative diptericin homologs, and 10 so-called edin (elevated-during-infection) proteins that might be involved in pathogen defense. Finally, we found four proline-rich peptides, including LSer-proline-rich peptide 4 (PRP4), which was strongly expressed in the crop.

The expression patterns of individual AMPs were complex and did not always correlate with AMP structure or family. For example, the expression of AMPs belonging to the attacin family was highly variable, both in terms of relative expression levels and tissue specificity. The most strongly expressed AMPs in the salivary gland and larval crop included one of the attacins (LSer-Atta2), a cecropin (LSer-Cec8), most of the defensins, several of the diptericins, and both sarcotoxins, suggesting that those AMPs might be secreted into wounds in larger amounts. The antifungal peptide lucimycin was expressed at lower levels in salivary glands than in the crop, but the highest expression levels were observed in the rest of the larval body, which predominantly comprises the fat body, the functional counterpart of the vertebrate liver.

Antibacterial activity of individual AMPs. Among the 47 AMPs identified in the *L. sericata* transcriptome, we selected 23 that could be successfully prepared as synthetic peptides. We measured the activities of the 23 selected AMPs against the Gram-negative bacterium *E. coli* in photometric growth inhibition assays, revealing MICs ranging from 0.8 to 6.2 μM for the cecropins and stomoxyns (Table 2). These AMPs also showed modest activity against the Gram-positive bacterium *M. luteus* in analogous photometric growth inhibition assays, revealing MICs ranging from 27 to 112 μM (Table 2). Although cecropins and cecropin-like peptides are only distantly related, the cecropin-like peptides also showed moderate activity against *M. luteus* and strong activity against *E. coli*. We found that the defensins were active against *M. luteus* but not *E. coli*, and only stomoxyn showed activity against *P.*

TABLE 2 MICs of *L. sericata* antimicrobial peptides for *E. coli* and *M. luteus*

Peptide	MIC (μM) (*n*) for[a]:	
	E. coli	*M. luteus*
LSer-AFP	>100	>100
LSer-Cec1	0.8 (2)	>100
LSer-Cec2	5.0 (6)	>100
LSer-Cec3	1.2 (2)	80.5 (5)
LSer-Cec4	1.1 (3)	112 (4)
LSer-Cec5	3.4 (3)	>100
LSer-Cec6	2.2 (9)	37.5 (7)
LSer-Def1	>100	>100
LSer-Def2	>100	>100
LSer-Def3	>100	16.4 (7)
LSer-Def4	>100	38.8 (14)
LSer-Def6	>100	51.6 (8)
LSer-Def7	>100	51.1 (6)
LSer-PRP1	>100	>100
LSer-PRP2	>100	>100
LSer-PRP3	>100	>100
LSer-PRP4	>100	>100
LSer-PCecL2	7.0 (2)	>100
LSer-PCecL3	7.6 (6)	>100
LSer-PCecL4	3.6 (10)	70.0 (5)
LSer-PCecL5	18 (8)	>100
LSerStomox1	6.2 (14)	27.5 (5)
LSerStomox2	6.2 (3)	37.5 (2)

[a] MIC values are the geometric means, and the number of experiments is given in parentheses.

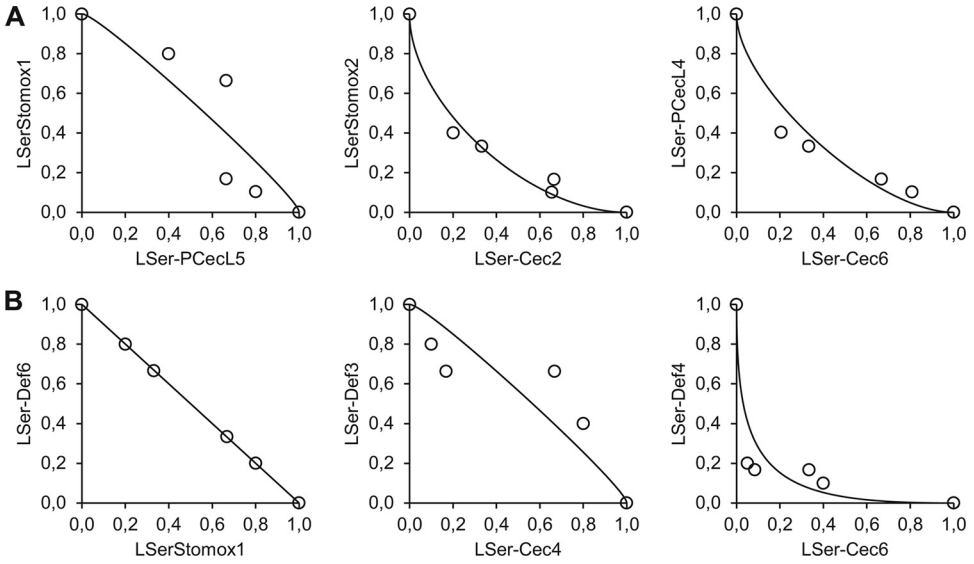

FIG 2 Representative isobolograms showing the interaction of two *L. sericata* antimicrobial peptides tested against *E. coli* (A) and *M. luteus* (B). A concave curve indicates synergy, and a straight line indicates additive effects.

aeruginosa DSM 50071 (MIC, 31 μM). None of the AMPs displayed activity against *S. aureus* within the concentration range tested. It should be noted that the salt content of the buffer can affect the activity of cationic AMPs, and both the LB and tryptic soy broth (TSB) media contain high concentrations of salt. However, these conditions are optimal for bacterial growth and also represent the typical environment of a wound, so they are suitable for testing the activity of AMPs intended for wound-healing applications.

Using an agar diffusion assay, the *L. sericata* cecropins, stomoxyns, cecropin-like, and proline-rich AMPs were tested against a panel of opportunistic Gram-negative pathogens, in addition to three Gram-positive pathogens (*Enterococcus faecalis* strain VRE 1528, *S. aureus* strain 511, and methicillin-resistant *S. aureus* [MRSA] strain 134/39), as well as the fast-growing *Mycobacterium smegmatis* strain 987, the filamentous fungus *Penicillium notatum* strain JP36, and the yeast *Candida albicans*. None of these AMPs were active against the Gram-positive pathogens *M. smegmatis*, *P. notatum*, and *C. albicans*. Very moderate activity was observed with two of the proline-rich AMPs against one strain of *E. coli* and one strain of *P. aeruginosa*. The cecropins and stomoxyns displayed pronounced anti-Gram-negative activities, with several of the AMPs inhibiting all strains tested, including those of *Enterobacter aerogenes*, *P. aeruginosa*, and *Staphylococcus gallinarum* (see Table S1 in the supplemental material). Activity against Gram-negative pathogens was also observed with the cecropin-like AMPs, although the spectrum of susceptible strains was less broad.

Combinatorial antibacterial activity of AMPs. Next, we tested pairs of AMPs in different ratios, again using *E. coli* and *M. luteus* photometric growth inhibition assays as representatives of Gram-negative and Gram-positive bacteria, respectively. The absolute MICs of the AMPs are summarized in Table 2. Typical isobolograms are shown in Fig. 2, and the sumFIC values for all AMP combinations are summarized in Table 3. Additive effects were observed when pairs of AMPs representing the stomoxyns, cecropins, or putative cecropin-like peptides were combined, as demonstrated by the straight line in the isobologram (Fig. 2) and sumFIC values of between 0.6 and 1.0 (Table 3). Moderate synergy against *M. luteus* was observed when the cecropin Cec6 was combined with the defensin Def4, as demonstrated by the concave curve in the isobologram (Fig. 2) and the mean sumFIC of 0.41 (Table 3). We also combined a proline-rich peptide, which was inactive against both *E. coli* and *M. luteus*, with an active cecropin, stomoxyn, or cecropin-like peptide. In this case, the active AMPs were not influenced by the presence of the inactive proline-rich peptide. A similar effect was observed when we combined the antifungal peptide lucimycin with an active cecropin, defensin, or stomoxyn (data not shown).

TABLE 3 Interactions of two *L. sericata* peptides against *E. coli* and *M. luteus*

Peptide combination	sumFIC (n)[a]	Interaction
E. coli		
PCecL5 + Stomox1	0.98 ± 0.25 (9)	Additive
Cec6 + PCecL4	0.82 ± 0.17 (11)	Additive
PCecL3 + Cec2	0.82 ± 0.22 (2)	Additive
PCecL5 + Cec6	0.85 ± 0.14 (2)	Additive
Cec2 + Stomox2	0.64 ± 0.04 (3)	Additive
Cec6 + Cec5	0.60 ± 0.17 (3)	Additive
PCecL4 + PCecL2	0.72 ± 0.10 (3)	Additive
M. luteus		
PCecL4 + Cec3	1.00 ± 0.00 (3)	Additive
PCecL4 + Def4	0.85 ± 0.17 (4)	Additive
Stomox1 + Def6	1.00 ± 0.00 (3)	Additive
Cec6 + Def4	0.41 ± 0.13 (15)	Synergistic
Cec4 + Def3	0.60 ± 0.00 (3)	Additive

[a] Values are means ± standard deviations from several experiments. The number of experiments is given in parentheses. The sumFIC results are interpreted as follows: a sumFIC of ≤0.5 indicates synergy, a sumFIC of >0.5 and ≤1 indicates additive effects, and a sumFIC of <2 indicates antagonism.

DISCUSSION

A comparative analysis of immunity-related transcriptomes from insects has revealed unexpected evolutionary plasticity (11). The spectrum of AMPs varies considerably between different insect species, from >50 in the invasive ladybird *Harmonia axyridis* (24) to a complete lack of known antibacterial peptides in the pea aphid *Acyrthosiphon pisum* (26). The diversity of AMPs probably reflects adaptations to different lifestyles, such as the colonization of habitats with either high or low microbial loads (11). In line with this hypothesis, we identified 47 genes in the *L. sericata* transcriptome that encode putative AMPs. This is the second largest repertoire of AMPs reported thus far in a multicellular organism and includes members of the attacin, cecropin/cecropin-like, defensin, diptericin, and proline-rich peptide families.

Our quantitative analysis of transcripts showed that almost all of the AMPs were expressed at higher levels in the rest of the body after the removal of salivary glands, crop, and gut than in samples from whole larvae. In addition to the integument, the remaining larval tissues mainly comprise the fat body, which is the major organ for AMP synthesis during systemic immune responses (27).

The number of *L. sericata* homologs representing distinct AMP families indicated the diversification of effector molecules. For example, the genome of the fruit fly *Drosophila melanogaster* carries only a single defensin gene, whereas we identified seven *L. sericata* defensins, suggesting that this species has benefited from several rounds of gene duplication and divergence. A similar expansion of the defensin repertoire was reported in the invasive ladybird *H. axyridis* (24). Defensins are cationic AMPs with six conserved cysteine residues that form three disulfide bonds. They comprise an amphipathic α-helix, an antiparallel β-sheet, and an N-terminal loop and form voltage-dependent channels (28). The majority of the *L. sericata* defensins were strongly expressed in the salivary glands, and four were active against *M. luteus*. This confirmed previous reports (9) but also revealed additional candidate defensins for wound-healing applications.

Attacins were first discovered in the silk moth *Hyalophora cecropia* and subsequently also in several *Diptera*. They are characterized by a molecular mass of 20 to 25 kDa, with a high glycine residue content (29). Attacins are active against some Gram-negative bacteria and were shown to inhibit the synthesis of outer bacterial membrane proteins (30). Proline-rich peptides are linear molecules that consist of 14 to 39 amino acid residues, of which >25% are proline, often arranged in triplets with basic residues, like arginine and histidine. Most proline-rich AMPs act selectively against Gram-negative bacteria but have little impact on Gram-positive bacteria (31).

We also identified several cecropins, cecropin-like peptides, and a stomoxyn that showed strong activity against *E. coli* and moderate activity against *M. luteus* but also against a spectrum of bacterial human pathogens. The cecropin family of peptides comprises linear amphipathic AMPs with an α-helical structure lacking cysteine residues. The first cecropin was discovered in the silk moth *H. cecropia* (32). Sarcotoxin is a cecropin homolog from the flesh fly *Sarcophaga peregrina* (33), and stomoxyns are cecropin homologs originally isolated from the stable fly *Stomoxys calcitrans* (34). They are primarily active against Gram-negative bacteria but show moderate activities toward Gram-positive bacteria, which is consistent with our results.

Pairwise testing of synthetic AMPs revealed mostly additive effects but provided evidence of synergy when we tested one combination of defensin and cecropin. Although testing individual AMPs can lead to the identification of broad microbial targets and MIC values for these specific peptides, most insects produce a broad spectrum of AMPs during innate immune responses, and synergic activity would reduce the quantity of each individual peptide required for effective protection against pathogens (11). Our data provide evidence for the simultaneous synthesis of numerous AMPs from different families, which are secreted into a wound at levels often too low to be detectable by traditional mass spectrometry. These findings suggest that lucifensin is unlikely to be the key antimicrobial component that protects the *L. sericata* larva from infection in a contaminated environment. Furthermore, lucifensin alone cannot explain the activity of the larval secretions against Gram-negative bacteria (15). A complex interaction of several AMPs might mediate the efficient antibacterial defense of the wound maggot.

The simultaneous synthesis of distinct AMPs that act in a synergistic or potentiating manner reduces the fitness costs associated with innate immunity, because lower quantities of individual AMPs are required for effective protection (35). Correspondingly, the therapeutic use of insect-derived AMPs will benefit from cooperative interactions that reduce the quantities required to achieve therapeutic effects. Our data provide the basis for further combinatorial studies with insect-derived AMPs, and we are currently producing the most potent *L. sericata* AMPs in larger quantities in order to test their therapeutic effects when applied to wounds individually or in combination. Our study identified a number of maggot-derived AMPs that can be produced in larger quantities required for topical therapeutic applications.

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REFERENCES

1. **Church JC.** 1996. The traditional use of maggots in wound healing, and the development of larva therapy (biosurgery) in modern medicine. J Altern Complement Med **2:**525–527. http://dx.doi.org/10.1089/acm.1996.2.525.
2. **Sherman RA, Hall MJ, Thomas S.** 2000. Medicinal maggots: an ancient remedy for some contemporary afflictions. Annu Rev Entomol **45:**55–81. http://dx.doi.org/10.1146/annurev.ento.45.1.55.
3. **Sherman RA.** 2003. Maggot therapy for treating diabetic foot ulcers unresponsive to conventional therapy. Diabetes Care **26:**446–451. http://dx.doi.org/10.2337/diacare.26.2.446.
4. **Sherman RA.** 2014. Mechanisms of maggot-induced wound healing: what do we know, and where do we go from here? Evid Based Complement Alternat Med **2014:**592419. http://dx.doi.org/10.1155/2014/592419.
5. **Nigam Y, Bexfield A, Thomas S, Ratcliffe NA.** 2006. Maggot therapy: the science and implication for CAM part I—history and bacterial resistance. Evid Based Complement Alternat Med **3:**223–227. http://dx.doi.org/10.1093/ecam/nel021.
6. **Nigam Y, Bexfield A, Thomas S, Ratcliffe NA.** 2006. Maggot therapy: the science and implication for CAM part II—maggots combat infection. Evid Based Complement Alternat Med **3:**303–308. http://dx.doi.org/10.1093/ecam/nel022.
7. **Bexfield A, Nigam Y, Thomas S, Ratcliffe NA.** 2004. Detection and partial characterisation of two antibacterial factors from the excretions/secretions of the medicinal maggot *Lucilia sericata* and their activity

against methicillin-resistant *Staphylococcus aureus* (MRSA). Microbes Infect **6:**1297–1304. http://dx.doi.org/10.1016/j.micinf.2004.08.011.
8. **Ratcliffe NA, Mello CB, Garcia ES, Butt TM, Azambuja P.** 2011. Insect natural products and processes: new treatments for human disease. Insect Biochem Mol Biol **41:**747–769. http://dx.doi.org/10.1016/j.ibmb.2011.05.007.
9. **Cerovsky V, Zdarek J, Fucik V, Monincova L, Voburka Z, Bem R.** 2010. Lucifensin, the long-sought antimicrobial factor of medicinal maggots of the blowfly *Lucilia sericata*. Cell Mol Life Sci **67:**455–466. http://dx.doi.org/10.1007/s00018-009-0194-0.
10. **Pöppel AK, Koch A, Kogel KH, Vogel H, Kollewe C, Wiesner J, Vilcinskas A.** 2014. Lucimycin, an antifungal peptide from the therapeutic maggot of the common green bottle fly *Lucilia sericata*. Biol Chem **395:**649–656. http://dx.doi.org/10.1515/hsz-2013-0263.
11. **Vilcinskas A.** 2013. Evolutionary plasticity of insect immunity. J Insect Physiol **59:**123–129. http://dx.doi.org/10.1016/j.jinsphys.2012.08.018.
12. **Altincicek B, Vilcinskas A.** 2007. Analysis of the immune-inducible transcriptome from microbial stress resistant, rat-tailed maggots of the drone fly *Eristalis tenax*. BMC Genomics **8:**326. http://dx.doi.org/10.1186/1471-2164-8-326.
13. **Vogel H, Badapanda C, Vilcinskas A.** 2011. Identification of immunity-related genes in the burying beetle *Nicrophorus vespilloides* by suppression subtractive hybridization. Insect Mol Biol **20:**787–800. http://dx.doi.org/10.1111/j.1365-2583.2011.01109.x.
14. **Schulenburg H, Boehnisch C.** 2008. Diversification and adaptive sequence evolution of *Caenorhabditis* lysozymes (*Nematoda*: *Rhabditidae*). BMC Evol Biol **8:**114. http://dx.doi.org/10.1186/1471-2148-8-114.
15. **Jaklic D, Lapanje A, Zupancic K, Smrke D, Gunde-Cimerman N.** 2008. Selective antimicrobial activity of maggots against pathogenic bacteria. J Med Microbiol **57**(Pt 5)**:**617–625. http://dx.doi.org/10.1099/jmm.0.47515-0.
16. **Valachová I, Bohová J, Pálošová Z, Takáč P, Kozánek M, Majtán J.** 2013. Expression of lucifensin in *Lucilia sericata* medicinal maggots in infected environments. Cell Tissue Res **353:**165–171. http://dx.doi.org/10.1007/s00441-013-1626-6.
17. **de la Paz Celorio-Mancera M, Heckel DG, Vogel H.** 2012. Transcriptional analysis of physiological pathways in a generalist herbivore: responses to different host plants and plant structures by the cotton bollworm, *Helicoverpa armigera*. Entomol Exper Appl **144:**123–133. http://dx.doi.org/10.1111/j.1570-7458.2012.01249.x.
18. **Vogel H, Heidel AJ, Heckel DG, Groot AT.** 2010. Transcriptome analysis of the sex pheromone gland of the noctuid moth *Heliothis virescens*. BMC Genomics **11:**29. http://dx.doi.org/10.1186/1471-2164-11-29.
19. **Vogel H, Wheat C.** 2011. Accessing the transcriptome: how to normalize mRNA pools, p 105–128. *In* Orgogozo V, Rockman MV (ed), Methods in molecular biology. Humana Press, New York, NY.
20. **Vogel H, Badapanda C, Knorr E, Vilcinskas A.** 2014. RNA-sequencing analysis reveals abundant developmental stage-specific and immunity-related genes in the pollen beetle *Meligethes aeneus*. Insect Mol Biol **23:**98–112. http://dx.doi.org/10.1111/imb.12067.
21. **Conesa A, Götz S.** 2008. Blast2GO: a comprehensive suite for functional analysis in plant genomics. Int J Plant Genomics **2008:**619832. http://dx.doi.org/10.1155/2008/619832.
22. **Myhre S, Tveit H, Mollestad T, Laegreid A.** 2006. Additional gene ontology structure for improved biological reasoning. Bioinformatics **22:**2020–2027. http://dx.doi.org/10.1093/bioinformatics/btl334.
23. **Kanehisa M, Goto S.** 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res **28:**27–30. http://dx.doi.org/10.1093/nar/28.1.27.
24. **Vilcinskas A, Mukherjee K, Vogel H.** 2013. Expansion of the antimicrobial peptide repertoire in the invasive ladybird *Harmonia axyridis*. Proc Biol Sci **280:**20122113. http://dx.doi.org/10.1098/rspb.2012.2113.
25. **Afonin S, Glaser RW, Berditchevskaia M, Wadhwani P, Gührs KH, Möllmann U, Perner A, Ulrich AS.** 2003. 4-fluorophenylglycine as a label for 19F NMR structure analysis of membrane-associated peptides. Chembiochem **4:**1151–1163. http://dx.doi.org/10.1002/cbic.200300568.
26. **Gerardo NM, Altincicek B, Anselme C, Atamian H, Barribeau SM, de Vos M, Duncan EJ, Evans JD, Gabaldon T, Ghanim M, Heddi A, Kaloshian I, Latorre A, Moya A, Nakabachi A, Parker BJ, Perez-Brocal V, Pignatelli M, Rahbe Y, Ramsey JS, Spragg CJ, Tamames J, Tamarit D, Tamborindeguy C, Vincent-Monegat C, Vilcinskas A.** 2010. Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. Genome Biol **11:**R21. http://dx.doi.org/10.1186/gb-2010-11-2-r21.
27. **Ferrandon D, Imler JL, Hetru C, Hoffmann JA.** 2007. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. Nat Rev Immunol **7:**862–874. http://dx.doi.org/10.1038/nri2194.
28. **Bonmatin JM, Bonnat JL, Gallet X, Vovelle F, Ptak M, Reichhart JM, Hoffmann JA, Keppi E, Legrain M, Achstetter T.** 1992. Two-dimensional ^{1}H NMR study of recombinant insect defensin A in water: resonance assignments, secondary structure and global folding. J Biomol NMR **2:**235–256. http://dx.doi.org/10.1007/BF01875319.
29. **Hultmark D, Engstrom A, Andersson K, Steiner H, Bennich H, Boman HG.** 1983. Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*. EMBO J **2:**571–576.
30. **Carlsson A, Engstrom P, Palva ET, Bennich H.** 1991. Attacin, an antibacterial protein from *Hyalophora cecropia*, inhibits synthesis of outer membrane proteins in *Escherichia coli* by interfering with *omp* gene transcription. Infect Immun **59:**3040–3045.
31. **Bulet P, Stocklin R.** 2005. Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept Lett **12:**3–11. http://dx.doi.org/10.2174/0929866053406011.
32. **Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG.** 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature **292:**246–248. http://dx.doi.org/10.1038/292246a0.
33. **Okada M, Natori S.** 1985. Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae. J Biol Chem **260:**7174–7177.
34. **Boulanger N, Munks RJ, Hamilton JV, Vovelle F, Brun R, Lehane MJ, Bulet P.** 2002. Epithelial innate immunity. A novel antimicrobial peptide with antiparasitic activity in the blood-sucking insect *Stomoxys calcitrans*. J Biol Chem **277:**49921–49926. http://dx.doi.org/10.1074/jbc.M206296200.
35. **Schmid-Hempel P.** 2005. Evolutionary ecology of insect immune defenses. Annu Rev Entomol **50:**529–551. http://dx.doi.org/10.1146/annurev.ento.50.071803.130420.