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Ixodes ricinus defensins attack distantly-related pathogens

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ABSTRACT

Antimicrobial peptides are ubiquitous components of eukaryotic innate immunity. Defensins are a well-known family of antimicrobial peptides, widely distributed in ticks, insects, plants and mammals, showing activity against bacteria, viruses, fungi, yeast and protozoan parasites. *Ixodes ricinus* is the most common tick species in Europe and is a vector of pathogens affecting human and animal health. Recently, six defensins (including two isoforms) were identified in *I. ricinus*. We investigated the evolution of the antimicrobial activity of *I. ricinus* defensins. Among the five unique defensins, only DefMT3, DefMT5 and DefMT6 showed in vitro antimicrobial activity. Each defensin was active against rather distantly-related bacteria ($P < 0.05$), significantly among Gram-negative species ($P < 0.0001$). These three defensins represent different clades within the family of tick defensins, suggesting that the last common ancestor of tick defensins may have had comparable antimicrobial activity. Differences in electrostatic potential, and amino acid substitutions in the β-hairpin and the loop bridging the α-helix and β-sheet may affect the antimicrobial activity in DefMT2 and DefMT7, which needs to be addressed. Additionally, the antimicrobial activity of the γ-core motif of selected defensins (DefMT3, DefMT6, and DefMT7) was also tested. Interestingly, compared to full length peptides, the γ-core motifs of these defensins were effective against less species of bacteria. However, the antifungal activity of the γ-core was higher than full peptides. Our results broaden the scope of research in the field of antimicrobial peptides highlighting the overlooked ability of arthropod defensins to act against distantly-related microorganisms.

1. Introduction

Antimicrobial peptides (AMPs) are evolutionarily ancient and highly diverse components of the innate immune system of many organisms. They have been isolated from diverse species including microbes, ticks, insects, fish, birds, plants and mammals, including humans (Rahnamaeian et al, 2015; Fogaça et al., 2004; Masso-Silva and Diamond, 2014; Nawrot et al., 2014; Rahnamaeian, 2011; Wang, 2014; Wiesner and Vilcinskas, 2010; Yu et al., 2006; Zhang and Sunkara, 2014; Zheng et al., 2012). Defensins are a well-characterised family of naturally occurring AMPs in many species, including ticks (Nakajima et al., 2002; Taylor, 2006). They have a well-conserved structure in which six cysteine residues form three disulfide bridges that are important for structural stability (Galay et al., 2012; Hajdusek et al., 2013; Hazlett and Wu, 2011; Pichu et al., 2009; Taylor, 2006). Most defensins are cationic, although some anionic peptides have been reported (Ganz and Lehrer, 1994;

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Lai et al., 2004; Tonk et al., 2014b).

Ticks are arthropods that cause direct damage by feeding and they also act as vectors for viruses, protozoa and bacteria (Bell-Sakyi et al., 2007). Ticks lack the highly-developed adaptive immune system found in vertebrates, but instead possess a primitive innate immune system that counteracts invading pathogens and maintains commensal microorganisms at controlled levels (Hajdusek et al., 2013). Tick defensins are active against both Gram-positive and Gram-negative bacteria, and also against intracellular rickettsia, protozoa and fungi (Bulet and Stocklin, 2005; Kocan et al., 2008; Tonk et al., 2014a, 2014b). The hard tick *Ixodes ricinus*, also known as the European castor bean tick, is the most common tick species in Europe (Crippa et al., 2002). *I. ricinus* is a vector for pathogens including *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, tick-borne encephalitis virus, louping ill virus and *Babesia* spp. (Biernat et al., 2014; Hudson et al., 1995; Rizzoli et al., 2014).

We recently reported six new defensins (DefMT2–7) in *I. ricinus*, expanding the number of known defensins in this tick species (Tonk et al., 2014b). Along with two previously reported *I. ricinus* defensins (Rudenko et al., 2007), these peptides form a highly diverse family. Studies on structure–function relationships of the defensins using several representative members (e.g. antibacterial arthropod defensins from *Tenebrio molitor*, *Pyrrhocoris apterus*, *Ornithodoros moubata* and *Stomoxys calcitrans* and mussel defensin from *Mytilus galloprovincialis* and antifungal plant defensin from *Raphous sativus*) have defined their functional region (Ahn et al., 2006; De Samblanx et al., 1997; Romestand et al., 2003; Varkey et al., 2006) primarily located in the C-terminal- β sheet domain, called the 'γ-core motif' (Yount and Yeaman, 2004).

Here we assessed the antimicrobial activity of DefMT2–7 against nine Gram-positive bacteria (*Listeria monocytogenes*, *L. fleischmannii*, *L. grayi*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *Staphylococcus aureus* and *S.epidermidis*), two Gram-negative bacteria (*Escherischia coli* and *Pseudomonos aeruginosa*) and the fungi *Fusarium culmorum* and *F. graminearum*. The antibacterial activity of the γ-core of selected defensins was not comparable to that of the whole peptide, while antifungal activity of the γ-core was higher than the activity of the whole peptide. Our results confirm that *I. ricinus* defensins are active against broad spectrum of bacteria and fungi, and have therefore evolved to protect ticks from diverse microbial pathogens.

2. Materials and methods

2.1. Defensin sequences and synthetic peptides

Publicly-available sequence information (Tonk et al., 2014b) was used to synthesise the mature peptides representing DefMT2 (GenBank accession number: JAA65352), DefMT3 (JAA71488), DefMT5 (JAA66832), DefMT6 (JAA71516) and DefMT7 (JAA69779) using solid phase peptide synthesis (SPPS) with ~ 95% purity (Pepmic, China). For purification and oxidation of the peptides, the crude product was purified by reverse phase HPLC (RP-HPLC) (Venusil XBP-C18, 4.6 × 250 mm), diluted without drying into folding buffer (1 M urea, 100 mM Tris, pH 8.0, 1.5 mM oxidised glutathione, 0.75 mM reduced glutathione, 10 mM methionine) and confirmed for complete oxidation using Ellman Reagent. The folded, fully oxidised peptide was further purified from the folding mixture by reverse phase HPLC (Venusil XBP-C18, 4.6 × 250 mm) and characterised by electrospray mass spectroscopy. Accordingly, the folded and oxidised peptide displayed a lower averaged molecular weight (data not shown). DefMT4 was not synthesised because the mature peptide is identical to DefMT3 (Tonk et al., 2014b). The γ-core motif of DefMT3, DefMT6 and DefMT7 was selected for testing antimicrobial activity. The amino acid sequences of DefMT3, DefMT6 and DefMT7 were aligned with the γ-core motif of Scapularisin-20 (Wang and Zhu, 2011) (Fig. 1) and the resultant γ-core motifs of DefMT3, DefMT6 and DefMT7 were synthesised by the service department of the Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic. The peptides with reduced cysteine residues were prepared and their purities were checked by RP-HPLC analysis to be more than 95% pure and identities were verified by mass spectrometry. Lyophilised peptides were stored at −20 °C.

2.2. Antibacterial assays

The peptides were prepared at concentrations of 0.015–250 μM and the minimum inhibitory concentrations (MICs) were determined against the Gram-positive bacteria *L. monocytogenes* (DSM 20600), *L. fleischmannii* (DSM 24998), *L. grayi* (DSM 20601), *L. marthii* (DSM 23813), *L. innocua* (DSM 20649), *L. welshimeri* (DSM 20650), *L. seeligeri* (DSM 20751), *S. aureus* (DSM 2569) and *S. epidermidis* (DSM 3269), and the Gram-negative species *E. coli* (D31) and *P. aeruginosa* (DSM 50071). The assays were carried out in 384-well plates (Griener Bio One, Frickenhausen, Germany) using Brain Heart Infusion Broth (BHIB) medium for *Listeria* spp. or Tryptic Soy Broth (TSB) (Roth, Karlsruhe, Germany) for the other bacteria. Cultures in the mid-logarithmic phase were used for growth inhibition assays. The initial optical density (OD_{600}) for *Listeria* spp. was set to 0.01 and for the rest of the bacteria to 0.001 to ensure full contact between each bacterial cell and the added defensins. Changes in OD_{600} values were monitored every 20 min for 24 h using an Eon Microplate Spectrophotometer (BioTel Instruments, VT, USA). Each assay also included a medium-only control. The "bacteriostatic effect" was evaluated by comparing the OD of the treated groups with that of the negative control (bacterium alone). When treated bacteria reached a growth plateau lower than that of the negative control we considered "bacteriostatic effect". The concentration of peptide at which such plateau was reached (i.e. further increases in peptide concentration did not decrease bacterial growth) was reported as the concentration with "bacteriostatic effect". Antimicrobial activity was tested against all bacteria and the assays were carried out at least twice with comparable results.

2.3. Antifungal assays

The fungicidal activity of the synthetic peptides was determined using an inhibition assay (Rahnamaeian et al., 2009). *F. culmorum* and *F. graminearum* strain 8/1 (Miedaner et al., 2000) were cultured in the dark on Nirenberg Synthetic Nutrient Agar (SNA) plates at 18 °C for 1–2 weeks (Rahnamaeian and Vilcinskas, 2012). Briefly, *F. culmorum* and *F. graminearum* were incubated with 0.1–20 μM of the peptides at room temperature for 24 h. Spore germination and growth were monitored using an inverted microscope (Motic AE21, Motic, China). The half maximal inhibitory concentration (IC_{50})

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Scapularisin-20  CGGFLKKTCICVMK 14
DefMT3           CGNFLKRTCICVKK 14
DefMT6           CSGIIKQTCTCYRK 14
DefMT7           CNGPFNIVCSCY-- 12
                 *       * *
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Fig. 1. γ-core motif of selected *I. ricinus* defensins. *I. ricinus* defensins aligned with γ-core motif of Scapularisin-20. γ-core motif sequences of selected defensins have 12 (DefMT7) and 14 amino acids (DefMT3, DefMT6). The conserved cysteine residues are indicated by stars.

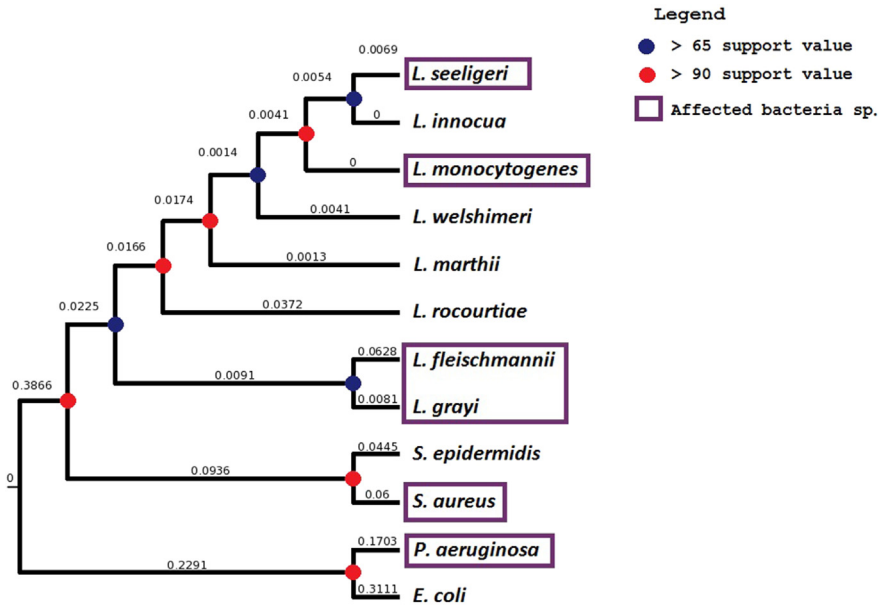

Fig. 2. Phylogenetic tree of the bacteria used in the antimicrobial assays. In this *16S rRNA* maximum likelihood phylogenetic tree, susceptible bacteria are boxed. Numbers on the branches represent branch lengths. Values of statistical support for internal branches are shown within circles.

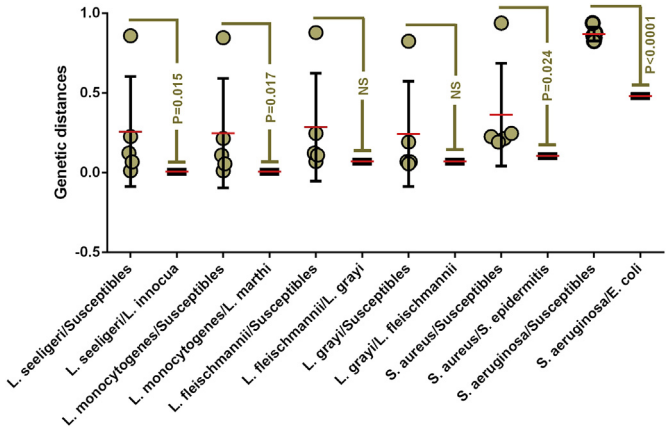

Fig. 3. *I. ricinus* defensins mainly affect distantly-related bacteria. The pairwise genetic distances (Table 3) of all the susceptible bacteria were compared to the closest relatives (susceptible or otherwise) using a ratio paired t-test. Susceptible bacteria and their closest relatives are presented in Table 4.

values were recorded when only 50% of the spores germinated (Germination means the outgrowth or emergence of hyphae from the polar heads of the spores).

2.4. Phylogenetic analyses

Phylogenetic trees were generated by aligning the amino acid sequences of 38 tick, 27 scorpion, 26 insect, 34 snake, 2 mollusc and 12 plant defensins (for the list of the species, see Supplemental Material 1 and for accession numbers, see Fig. 4). A bacterial phylogenetic tree was created using the following *16S rRNA* sequences (accession numbers in brackets): *L. monocytogenes* (JF967618), *L. fleischmannii* (JN093103), *L. grayi* (JF967632), *L. marthii* (EU545983), *L. innocua* (AL596173), *L. welshimeri* (JF967630), *L. seeligeri* (DQ065845), *L. rocourtiae* (FJ557241), *S. aureus* (DQ269498) and *S. epidermidis* (KF993665), *E. coli* (AB045731) and *P. aeruginosa* (EU381200). The sequences were aligned using MAFFT (v7), configured for the highest accuracy (Katoh and Standley, 2013). Ambiguous regions were removed with Gblocks (v 0.91b) (Castresana, 2000). The phylogenetic trees were reconstructed using the maximum likelihood method implemented in PhyML v3.0 aLRT (Anisimova and Gascuel, 2006; Guindon and Gascuel, 2003). The reliability for the internal branches was assessed using the approximate likelihood ratio test (aLRT – SH-Like) (Anisimova and Gascuel, 2006). The trees were visualised and edited using EvolView (Zhang et al., 2012).

2.5. Statistical analysis of relatedness

Null models that randomised the tips of the trees were used to compare the relatedness among defensins and the bacterial phylogenetic tree. Standardised effect sizes of the phylogenetic structure were calculated for mean pairwise distances and mean nearest taxon distances by comparing the observed phylogenetic relatedness to the pattern expected under a null model of phylogeny or community randomisation. Standardised effect sizes describe the differences between phylogenetic distances in the observed communities versus null communities generated by randomisation, divided by the standard deviation of the phylogenetic distances in the null data. We used Picante for these calculations and to plot the resulting dendrograms (Kembel et al., 2010).

2.6. Statistical analysis of genetic distances

Genetic distances among the bacteria were determined by pairwise comparisons. The pairwise genetic distances between the susceptible bacteria were compared to those of each closest relative (susceptible or otherwise). We used the ratio paired t-test (GraphPad Prism v6, GraphPad Software Inc.) to average the logarithm of the ratio of the genetic distances and to evaluate the null hypothesis. (i.e. the defensins in this study are active against closely related pathogens). In this test the mean of logarithms is zero with 95% confidence.

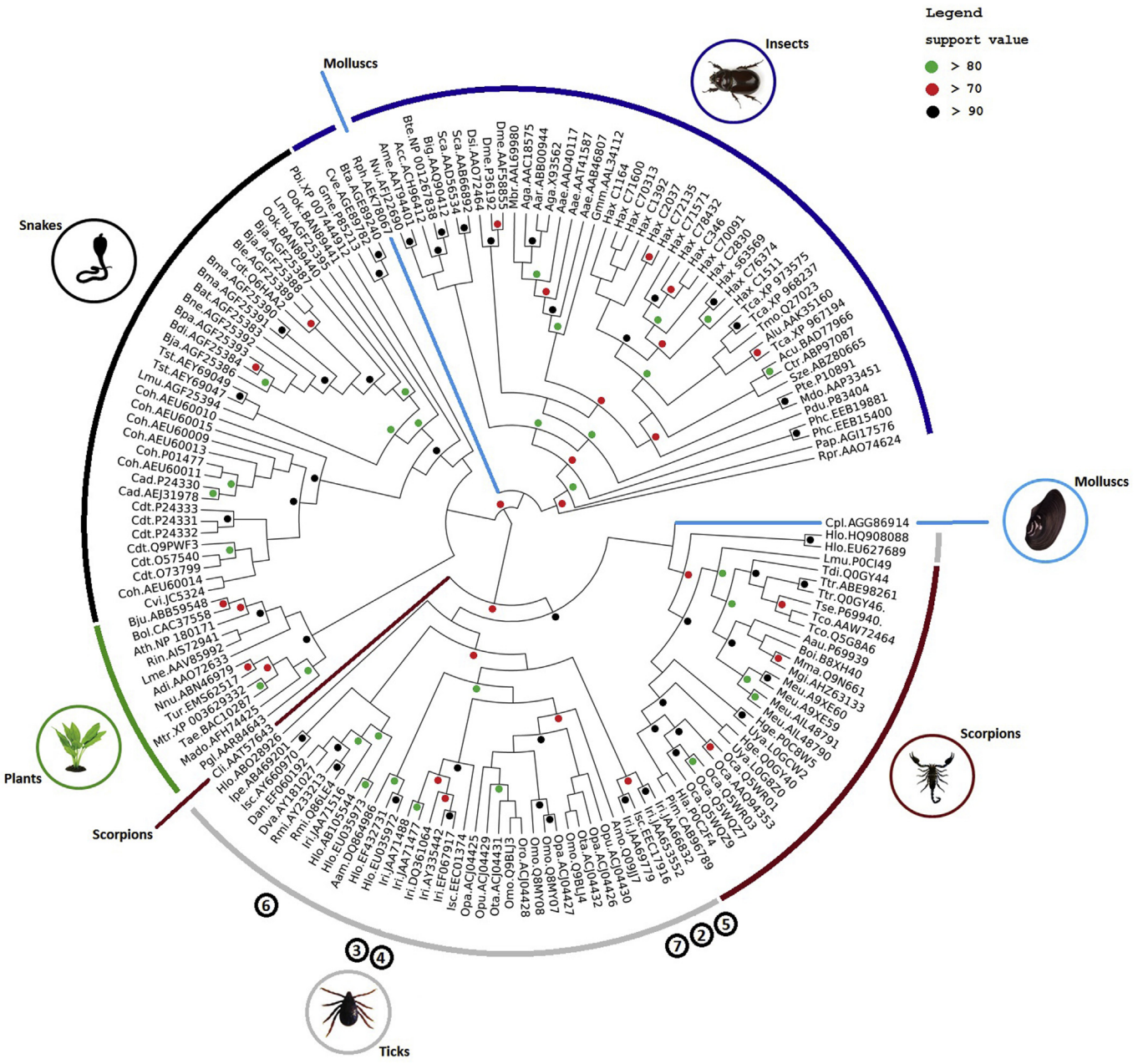

Fig. 4. Phylogenetic tree of defensins. The maximum likelihood phylogenetic tree was built using amino acid sequences of defensins from ticks, scorpions, snakes, plants, insects and molluscs. The *I. ricinus* defensins included in this study are identified with numerals. The code for the name of the species is provided in Supplementary Material 1.

2.7. Computational biology

The Robetta server (http://robetta.bakerlab.org/) was used for the tertiary modelling of *I. ricinus* defensins DefMT2–7. Each modelled structure was prepared using the Schrodinger's Maestro (Schrödinger, 2014) package Protein Preparation Wizard and then minimised to remove any steric clashes. The electrostatic potential for each structure was calculated using the Possion-Boltzman equation (Schrödinger, 2014) implemented in the Maestro package.

3. Results

3.1. Antimicrobial activity of I. ricinus defensins

DefMT3, DefMT5 and DefMT6 had bactericidal effect on four species of *Listeria* (*L. monocytogenes*, *L. fleischmannii*, *L. grayi* and *L. seeligeri*) as well as *S. aureus* and also the Gram-negative bacterium *P. aeruginosa*. In addition, DefMT6 showed bacteriostatic effects against the Gram-positive species *S. epidermidis* and the Gram-negative species *E. coli* (Table 1). The antibacterial activity of the γ-cores of DefMT3, DefMT6 and DefMT7 was not comparable to that of the whole peptides (Table 1). Only the γ-core of DefMT6 showed bacteriostatic effect at lower concentration (30 μM) than that of the whole peptide (120 μM) (Table 1). While the γ-cores of DefMT3 and DefMT7 did not show bactericidal or bacteriostatic effect.

DefMT3, DefMT5 and DefMT6 showed antifungal activity against *F. culmorum* and *F. graminearum*. Both DefMT3 and DefMT5 completely inhibited the germination of *F. culmorum* and *F. graminearum* spores at 4 μM, whereas DefMT6 inhibited the

Table 1
Minimum inhibitory concentrations (MICs) of *I. ricinus* defensins and γ-core of selected defensins.

Bacterial species	MIC (μM)							
	DefMT2	DefMT3	γ-core DefMT3	DefMT5	DefMT6	γ-core DefMT6	DefMT7	γ-core DefMT7
L. monocytogenes (Gr^+)	-	15	-	1	0.05	-	-	-
L. fleischmannii (Gr^+)	-	60	-	15	1	-	-	-
L. grayi (Gr^+)	-	8	60[a]	8	2	30[a]	-	-
L. marthii (Gr+)	-	-	-	-	-	-	-	-
L. innocua (Gr^+)	-	-	-	-	-	-	-	-
L. welshimeri (Gr^+)	-	-	-	-	-	-	-	-
L. seeligeri (Gr^+)	-	15	-	15	0.5	-	-	-
S. aureus (Gr^+)	-	60	-	15	2	-	-	-
S. epidermidis (Gr^+)	-	-	-	-	120[a]	30[a]	-	-
E. coli (Gr^-)	-	-	-	-	120[a]	-	-	-
P. aeruginosa (Gr^-)	-	30	-	15	2	-	-	-

Gr^+: Gram-positive.
Gr^-: Gram-negative.
-: *No effect.*
[a] Bacteriostatic effect.

germination of both species at 2 μM (Table 2). DefMT2 and DefMT7 showed no antibacterial or antifungal effects. The γ-cores of DefMT3 and DefMT6, but no DefMT7, were more active than the whole peptides (Table 2). DefMT3 totally inhibited the spore germination of *F. culmorum* and *F. graminearum* at 1 and 2 μM, respectively (Table 2). In the case of DefMT6, maximum activity was recorded at 2 μM and 5 μM against *F. culmorum* and *F. graminearum*, respectively (Table 2).

Our bacterial phylogenetic tree revealed the relationship between the susceptible bacteria and their closest relatives (Fig. 2). The relatedness between defensin activity and the bacterial phylogeny was 0.04, indicating there is little correlation between defensin activity and the phylogenetic relationships among the affected bacteria. We therefore calculated the pairwise genetic distances among all the susceptible bacteria (Table 3) compared to their closest relatives (Table 4). Interestingly, all the susceptible bacteria were distantly related ($P < 0.05$), except for *L. grayi* and *L. fleischmannii* (Fig. 3). This demonstrated that DefMT3, DefMT5 and DefMT6 affect a wide range of unrelated microorganisms. Interestingly, DefMT3, DefMT5 and DefMT6 are distantly related to each other. Indeed, they belong to different phylogenetic clusters within the tick defensin family (Fig. 4). This suggests their activity against distantly-related microorganisms may have emerged independently in the three clusters. However, the most parsimonious explanation is that this property was present in the last common ancestor of all three defensins.

Table 2
Antifungal activity of *I. ricinus* defensins and γ-core of selected defensins.

Peptide	IC_{50} value (μM)	
	Fungi	
	F. culmorum	*F. graminearum*
DefMT2	-	-
DefMT3	4	4
γ-core DefMT3	1	2
DefMT5	4	4
DefMT6	12	2
γ-core DefMT6	2	4
DefMT7	-	-
γ-core DefMT7	-	-

IC_{50} value: Half maximal inhibitory concentration.
-: **No effect.**

3.2. Structural basis for the absence of DefMT2 and DefMT7 antimicrobial activity

The modelled structures of the *I. ricinus* defensins show that they are highly conserved and belong to the CS-α/β-defensin subfamily, featuring the archetypal three disulfide bridges (Fig. 5). The absence of DefMT7 antimicrobial activity may reflect its electrostatic potential, which is much more anionic that the other *I. ricinus* defensins (Supplemental Material 2). Both DefMT2 and DefMT5 are highly conserved at the amino acid level (Fig. 5), but the former did not affect the microbial pathogens we tested (Tables 1 and 2). There is a mutation in the loop bridging the α-helix and β-sheet (highlighted in grey in Fig. 5) that may account for the functional differences between DefMT2 and DefMT5. Furthermore, the Trp24 indole group in DefMT2 is acidic compared to the basic properties of Arg, which occupies the equivalent position in DefMT3, DefMT5 and DefMT6. There is a further Arg → His substitution in DefMT2 between the second and third cysteine residues (in the α-helix) that may also explain the lack of antimicrobial activity. The electrostatic differences caused by this mutation are summarised in Supplemental Material 2. Furthermore, DefMT6 contains mutations in the β-hairpin, specifically residue Ile32 (highlighted in green in Fig. 5), that may explain the additional bacteriostatic effect against *S. epidermidis* and *E. coli* (Table 1).

4. Discussion

Defensins have been discovered in hard and soft ticks (Johns et al., 2001; Nakajima et al., 2001; Saito et al., 2009; Tonk et al., 2014b; Wang and Zhu, 2011). Defensins DefMT2–7 are representative of the diversity found in the *I. ricinus* defensin family (Tonk et al., 2014b). We excluded DefMT4 from this study because DefMT3 and DefMT4 are isoforms that share 98% identity at the nucleotide and protein levels and they have identical mature peptide sequences, suggesting they have the same activity (Tonk et al., 2014b). We showed that three of the five unique defensins have antimicrobial activity and that they are predominantly active against the same pathogens; with the bacteriostatic effect of DefMT6 the only exception. Redundancy is a common feature of the animal immune system (Buchmann, 2014; Haase et al., 2014). Redundancy in immune components contributes to the robustness of the system. Interestingly, the three defensins with antimicrobial activity (DefMT3, DefMT5 and DefMT6) were expressed in different tick tissues. DefMT3 is ubiquitous, DefMT5 is only found in salivary glands and DefMT6 is present in the salivary glands, ovaries and

Table 3
Pairwise comparison of genetic distances among the bacteria used in the antimicrobial assays.

Bacteria spp.	*L. seeligeri*	*L. innocua*	*L. monocytogenes*	*L. welshimeri*	*L. marthii*	*L. rocourtiae*	*L. fleischmannii*	*L. grayi*	*S. epidermidis*	*S. aureus*	*P. aeruginosa*	*E. coli*
L. seeligeri		0,0068	0,0122	0,0204	0,0190	0,0723	0,1237	0,0689	0,2124	0,2278	0,8601	1,0009
L. innocua			0,0054	0,0135	0,0121	0,0654	0,1168	0,0621	0,2055	0,2209	0,8533	0,9941
L. monocytogenes				0,0081	0,0067	0,0600	0,1114	0,0567	0,2001	0,2155	0,8479	0,9887
L. welshimeri					0,0067	0,0600	0,1114	0,0567	0,2001	0,2155	0,8479	0,9887
L. marthii						0,0559	0,1073	0,0526	0,1960	0,2114	0,8438	0,9846
L. rocourtiae							0,1257	0,0710	0,2144	0,2298	0,8622	1,0030
L. fleischmannii								0,0709	0,2325	0,2479	0,8803	1,0211
L. grayi									0,1778	0,1932	0,8256	0,9664
S. epidermidis										0,1045	0,9241	1,0649
S. aureus											0,9395	1,0803
P. aeruginosa												0,4814
E. coli												

Table 4
Closest relative of the susceptible bacteria.

Susceptible bacteria	Closest relative
L. seeligeri	*L. innocua*
L. monocytogenes	*L. marthii*
L. fleischmannii	*L. grayi*
S. aureus	*S. epidermidis*
P. aeruginosa	*E. coli*

Malpighian tubules (Tonk et al., 2014b).

Defensins are primarily active against Gram-positive bacteria, causing cell lysis by inducing the formation of membrane-penetrating channels (Gillespie et al., 1997). We found that *I. ricinus* defensins have a wide spectrum of antimicrobial activity, showing activity not only against Gram-positive and Gram-negative bacteria, but also against fungi. The broad antimicrobial activity of tick defensins has been reported, e.g. *I. persulcatus* defensin is active against Gram-positive and Gram-negative bacteria (Saito et al., 2009), *Ds*-defensin from *Dermacentor silvarum* is active against Gram-positive bacteria, Gram-negative bacteria and fungi (Wang et al., 2015), and the soft tick *O. moubata* defensin, Isoform A, is active against Gram-positive bacteria (Nakajima et al., 2003). However, this is the first study showing that tick defensins are effective against distantly-related pathogens. We calculated pairwise distances to determine the phylogenetic proximity of the pathogens. We observed that affected pathogens were not closely related, which is counterintuitive because one would expect that susceptibility to antimicrobial compounds (defensins in this case) would be found in closely-related microorganisms. In this sense, *I. ricinus* defensins behave like broad-spectrum antibiotics. Interestingly, whereas *I. ricinus* defensins were active against distantly related pathogens, the defensin Scapularisin-6 from the closely-related species *I. scapularis* is only active against the Gram-positive bacterium *L. grayi* and fungi, but not against Gram-negative bacteria or any other *Listeria* species (Tonk et al., 2014a).

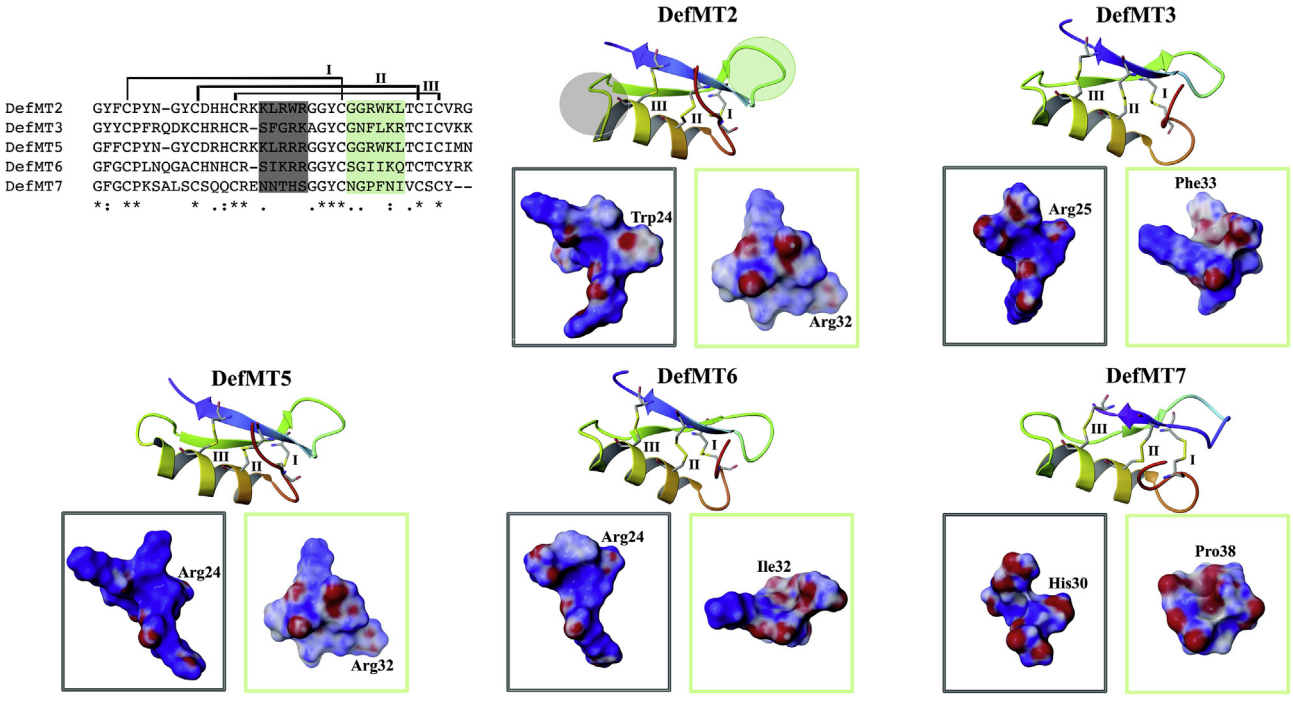

Fig. 5. Tertiary structures of *I. ricinus* defensins. The alignment (left panel) shows the conserved cysteine residues forming the three disulfide bridges (roman numerals) and the two loop regions highlighted in grey and green. The tertiary structures are colour-coded from the N-terminus (red) to the C-terminus (blue). Below each structure, the electrostatic potential of the loop regions (blue = positive; white = neutral; red = negative) corresponds to the highlighted regions in the structures and alignment. The orientation was adjusted to visualise the specific residues more clearly. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Previous studies have shown that the functional region of defensins is primarily located in the C-terminal β-sheet domain, called the 'γ-core motif' (Wang and Zhu, 2011). In total, twenty-five defensins were found with significant sequence similarity to Scapularisin, named Scapularisins −1 to −25, from *I. scapularis* (Hynes et al., 2005). Most of the *I. scapularis* defensins contain a complete γ-core of 11 or 14 amino acids with a GXC motif. A fragment corresponding to the γ-core of Scapularisin-20 showed slightly more effect on Gram + bacteria than on Gram-bacteria (Wang and Zhu, 2011). Several synthetic γ-core-derived peptides from tenecin 1, mussel defensin, and navidefensin2-2 were found to possess a wide spectrum of antimicrobial activity (Gao and Zhu, 2010; Romestand et al., 2003). Our results supports the hypothesis that the activity of defensins is mainly in the γ-core because γ-core motifs of DefMT3 and DefMT6 showed higher antifungal activity compare to whole peptides, additionally bacteriostatic effect of γ-core motif of DefMT6 was more effective on *S. epidermidis*, compare to whole DefMT6. However, the bactericidal effect of the γ-core of DefMT3 and DefMT6 was reduced compared to the full peptides. This suggests that a different motif (*i.e.* not the γ-core) may be responsible for the bactericidal activity of *I. ricinus* defensins.

DefMT7 is the only anionic *I. ricinus* defensin. The remainder are cationic, which is thought to be necessary for their activity (Sagaram et al., 2011). Anionic and cationic antimicrobial peptides may have different effects; therefore DefMT7 may act against a distinct range of pathogens compared to the other defensins although not to the bacteria and fungi we tested. Most antimicrobial peptides have an amphipathic structure (α-helix, β-hairpin-like β-sheet, β-sheet or α-helix/β-sheet mixture) that may be necessary for their antimicrobial effects (Bulet et al., 2004). Plant and mussel defensins also possess antimicrobial and antifungal activity and this has been attributed to the β-hairpin loop (De Samblanx et al., 1996; Romestand et al., 2003). In *Drosophila melanogaster* defensin drosomycin, the α-helix, β-hairpin loop and α-helix/β-sheet loop (known as the α-patch, γ-patch and m-loop) are responsible for its activity (Zhang and Zhu, 2009). DefMT2 and DefMT7 did not show activity against the microbes we tested but this does not exclude the possibility that they act against other species or require specific condition(s) to be able to exhibit their activity (Rahnamaeian and Vilcinskas, 2015). Alternatively, these defensins may have toxic functions as suggested before (Cabezas-Cruz and Valdes, 2014). In agreement with this, our phylogenetic tree of defensins shows that tick defensins are closely related to scorpion defensins and may share a common ancestor. Interestingly, scorpion defensins have been reported to exhibit both toxic and antimicrobial activities (Diego-Garcia et al., 2008).

5. Conclusion

Five *I. ricinus* defensins were functionally characterised and three showed activity against Gram-positive, Gram-negative bacteria and fungi, whereas two showed no activity against our panel of test organisms. Our results reveal that the mature *I. ricinus* defensins have the capacity to affect distantly-related bacteria and fungi, making them useful as candidates for the development of novel anti-infective or anti-fungal drugs. Interestingly, the γ-cores of selected *I. ricinus* defensins were active against less bacteria species compared to full length peptides.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2015.08.001.

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