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The functional interaction between abaecin and pore-forming peptides indicates a general mechanism of antibacterial potentiation

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ABSTRACT

Long-chain proline-rich antimicrobial peptides such as bumblebee abaecin show minimal activity against Gram-negative bacteria despite binding efficiently to specific intracellular targets. We recently reported that bumblebee abaecin interacts with *Escherichia coli* DnaK but shows negligible antibacterial activity unless it is combined with sublethal doses of the pore-forming peptide hymenoptaecin. These two bumblebee peptides are co-expressed in vivo in response to a bacterial challenge. Here we investigated whether abaecin interacts similarly with pore-forming peptides from other organisms by replacing hymenoptaecin with sublethal concentrations of cecropin A (0.3 μM) or stomoxyn (0.05 μM). We found that abaecin increased the membrane permeabilization effects of both peptides, confirming that it can reduce the minimal inhibitory concentrations of pore-forming peptides from other species. We also used atomic force microscopy to show that 20 μM abaecin combined with sublethal concentrations of cecropin A or stomoxyn causes profound structural changes to the bacterial cell surface. Our data indicate that the potentiating functional interaction between abaecin and pore-forming peptides is not restricted to specific co-expressed peptides from the same species but is likely to be a general mechanism. Combination therapies based on diverse insect-derived peptides could therefore be used to tackle bacteria that are recalcitrant to current antibiotics.

1. Introduction

The insect innate immune system comprises a complex series of cellular and molecular defense strategies that recognize and combat pathogens [20,31]. Comparative genomics and transcriptomics has revealed remarkable levels of evolutionary plasticity among the components, which allows insects to respond more rapidly to pathogens with smaller genomes and shorter life cycles, and thus a greater capacity for evolutionary adaptation [5,6,31]. One particularly adaptable component of the insect immune system is the spectrum of antimicrobial peptides (AMPs) that can be deployed, a diversification process driven by gene duplication, exon duplication and exon shuffling [30]. Larger repertoires of AMPs not only target different pathogens through specific interactions, but also allow functional interactions among different AMPs, such as potentiation and synergism [21]. This can extend the range and specificity of responses and can also boost the efficacy of AMPs at low concentrations, which helps to conserve resources and reduce the trade-off between immunity and other functions [27].

We recently reported that functionally-distinct insect AMPs expressed concurrently during an immune response can reciprocally potentiate each other's activities [21]. Similar phenomena have been reported in other contexts [9,11,28,36,37]. However, such interactions have not been studied in detail and the evaluation of AMPs in terms of potency against different pathogens is almost universally achieved using individual peptides, which is likely to underestimate their therapeutic potential.

Long-chain (>20 residues) proline-rich antimicrobial peptides were originally believed to be active solely against Gram-positive bacteria and fungi [19,22,23]. We recently found that bumblebee abaecin is also inactive against Gram-negative bacteria such as *Escherichia coli* at higher concentrations [21]. Interestingly, we

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also found that much lower concentrations of abaecin were lethal to *E. coli* in the presence of the pore-forming peptide hymenoptaecin, another bumblebee AMP which is co-expressed in vivo with abaecin [26]. We observed evidence for reciprocal potentiation between these AMPs, i.e. hymenoptaecin created membrane pores that allowed abaecin access to its intracellular target (DnaK) and abaecin inhibited protein metabolism and prevented the bacteria from repairing the pores, thus reducing the minimal inhibitory concentration (MIC) of both components [21].

Although this earlier study provided clear evidence for potentiating interactions among AMPs, both peptides are co-expressed naturally in response to bacterial challenge so it remained unclear whether this was a specific mechanism or an example of a more general phenomenon. Here we describe further experiments in which abaecin is combined with pore-forming peptides from two heterologous species. We used a β-galactosidase assay to determine the permeability of the bacterial membrane in the presence of different combinations and quantities of each AMP, and atomic force microscopy (AFM) to investigate the impact of different AMP combinations on the structure of the bacterial cell surface. The ability to combine functionally compatible AMPs from different insect species could expand the spectrum of pathogens that can be targeted with peptide antibiotics and could usher in a new era of combinatorial microbicides.

2. Materials and methods

2.1. Peptide synthesis and modification

Abaecin (*Bombus pascuorum*), cecropin A (*Hyalophora cecropia*) and stomoxyn (*Stomoxys calcitrans*) peptides were synthesized by PANATecs (Tübingen, Germany) with >95% purity and the C-termini of cecropin A and stomoxyn were amidated. The amino acid sequences of the three peptides are shown in Table 1.

2.2. Growth inhibition assays

Growth inhibition assays in 384-well plates (Griener Bio One, Frickenhausen, Germany) were carried out using *E. coli* strain D31 in lysogeny broth (starting $OD_{600} = 0.001$). The AMPs were added as serial dilutions from 250 to 0.03 μM (final concentrations). The OD_{600} was measured over 16 h with a reading every 20 min in an Eon™ Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA). Control cultures with no AMPs were included in the assays.

2.3. Permeabilization assays

The permeabilization activities of the AMPs were determined by measuring β-galactosidase activity in *E. coli* strain JM83. This contains the plasmid pCH110 (Pharmacia-Amersham, Piscatway, NJ, USA), which confers ampicillin resistance and the constitutive synthesis of cytoplasmic β-galactosidase [37]. The AMPs were pre-incubated for 15 min at 37 °C in 23 μl 20 mM phosphate buffer (pH 6.8) before adding 2 μl of the mid-logarithmic phase bacterial cell suspension (5×10^5 colony forming units) prepared in the same buffer. The samples were incubated at 37 °C for 45 min before adding 220 μl 20 mM HEPES/150 mM NaCl (pH 7.5) and 5 μl 50 mM aqueous *p*-nitrophenyl-β-D-galactopyranoside. After incubation for 90 min at 37 °C, the absorbance was measured at 405 nm in Benchmark Plus™ Microplate Spectrophotometer (BioRad Laboratories, Hercules, CA, USA). Samples containing bacteria incubated without AMPs were used as negative controls and samples of bacteria killed with 5 μM synthetic cecropin B (Sigma–Aldrich, St. Louis, MO, USA) were used as positive controls. The permeabilization index was calculated by correcting for the negative control at 0% and setting the positive control to 100%. All assays were carried out three times in triplicate. Statistical analysis was carried out using Student's *t*-test with the following significance values: $^{*}p < 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

2.4. Atomic force microscopy

Samples for AFM were prepared as previously described [36,37]. Log-phase *E. coli* JM83 cells in 100 μl lysogeny broth ($OD_{600} = 0.2$) were incubated in the presence of AMPs (or without AMPs as a negative control) at 37 °C for 90 min. The final concentrations of abaecin, cecropin A, and stomoxyn were 20 μM, 0.3 μM and 0.05 μM, respectively. The bacteria were then centrifuged (8000 × *g*, 10 min, 4 °C), washed twice with apyrogenic water, suspended in 5 μl of apyrogenic water, applied to mica disks and dried overnight at 28 °C before analysis.

Cell surface imaging and analysis were carried out using a NanoScope V AFM (Veeco Instruments, Plainview, NY, USA) in Peak Force QNM operation mode, fitted with a silicon tip NSG 30 with a spring constant of 20 N/m (NT-MDT, Moscow, Russian Federation). Three fields were imaged for each sample. Data were analyzed using Nanoscope Analysis v1.40 (Veeco Instruments). The average surface root mean square (RMS) roughness was calculated from 25 fields (300 nm × 300 nm) measured over the entire cell surface in 3 μm × 3 μm areas. WSxM v5.0 software was used to produce 3D images and section profiles. Statistical differences in RMS roughness were determined by analysis of variance (ANOVA) using Fisher's least significant difference (LSD) test.

2.5. Analysis of hydropathy

The hydropathy of the pore-forming AMPs was determined using ProtScale [14] on the ExPASy server (http://web.expasy.org/protscale/).

3. Results

3.1. Potency of the AMPs in bacterial growth inhibition assays

E. coli strain D31 showed no susceptibility in the presence of up to 200 μM abaecin and entered the exponential growth phase ~4 h after the initiation of cultivation (data not shown). In contrast, cecropin A and stomoxyn exhibited potent bactericidal activities at concentrations of 1 and 0.1 μM, respectively. The sublethal concentrations of the AMPs were determined by preparing serial dilutions and repeating the growth inhibition assays. This revealed that cecropin A and stomoxyn had sublethal concentrations of 0.3 and 0.05 μM, respectively (data not shown). Combinatorial assays were carried out by supplementing the sublethal doses of each pore-forming peptide with 20 μM abaecin (Fig. 1).

3.2. Permeabilization of the bacterial cell membrane

To determine whether the lethal effect of the combined peptides was due to the increase in the permeabilization of the bacterial membrane, we used the standard bacterial strain *E. coli* JM83 which constitutively expresses cytoplasmic β-galactosidase. Both cecropin A and stomoxyn caused near maximum permeabilization of the bacterial membrane at a concentration of 2.5 μM (Fig. 2). The administration of sublethal concentrations of each peptide individually had no effect on membrane permeability, but when each was supplemented with 20 μM abaecin (which alone increased permeability by only ~ 5%), the permeabilization of the bacterial membrane increased significantly. The presence of 0.3 μM cecropin A and 0.05 μM stomoxyn increased membrane permeability caused by 20 μM abaecin by 7-fold and 2-fold, respectively (Fig. 2). These

Table 1
Amino acid sequences of AMPs used in this study. The C-terminus of cecropin A and stomoxyn was modified by amidation.

Peptide	Origin	Sequence	Reference
Abaecin	Bombus pascuorum	FVPYNPPRPGQSKPFPSFPGHGPFNPKIQWPYPLPNPGH	[25]
Cecropin A	Hyalophora cecropia	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2	[29]
Stomoxyn	Stomoxys calcitrans	RGFRKHFNKLVKKVKHTISETAHVAKDTAVIAGSGAAVVAAT-NH_2	[4]

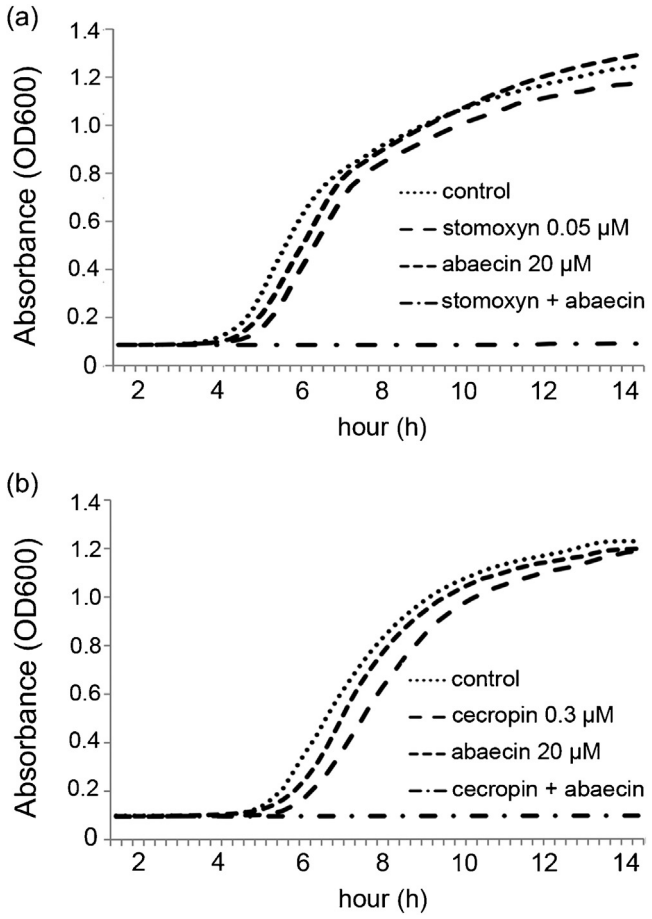

Fig. 1. *Escherichia coli* growth inhibition assays. (a) *E. coli* strain D31 in mid-logarithmic phase was incubated with medium (control) or with abaecin and stomoxyn, alone or in combination. (b) *E. coli* strain D31 in mid-logarithmic phase was incubated with medium (control) or with abaecin and cecropin A, alone or in combination. The growth rate was determined by measuring the optical density (OD) of the culture at 600 nm.

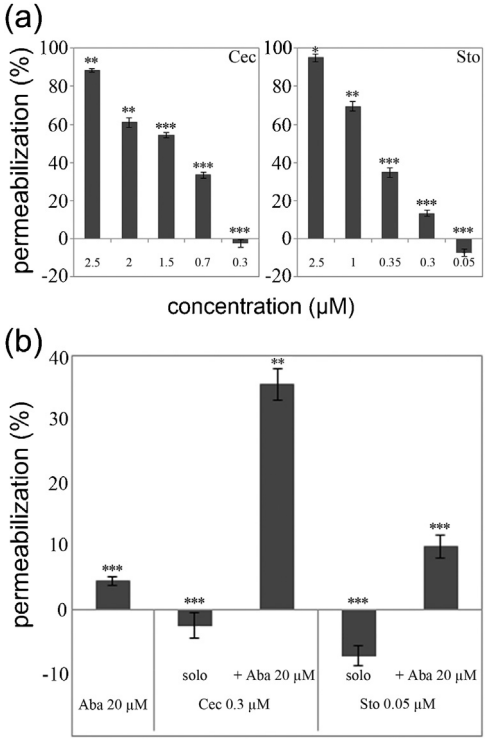

Fig. 2. Membrane permeabilization assay in *E. coli* JM83 cells. The graphs show the activities of abaecin (Aba), cecropin A (Cec) and stomoxyn (Sto) against the bacterial cell envelope determined by measuring activity of β-galactosidase. Cells in mid-logarithmic phase were treated with peptides alone or in combination, and the absorbance at 405 nm was proportional to the amount of the substrate converted by the β-galactosidase. Living bacteria incubated with no AMPs were used as a negative control and bacteria killed by treatment with 5 μM synthetic cecropin B were used as a positive control. Values represent means ± SD ($n = 3$). Statistical significance versus control: $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

results clearly show that the two heterologous pore-forming peptides potentiate the activity of the long-chain proline-rich peptide abaecin in *E. coli*, even though they have no pore-forming ability when applied individually at their sublethal concentrations. The permeabilization assay results clearly correlated with the results of growth inhibition assay (Fig. 1).

3.3. Impact of AMPs on the structure of the bacterial cell surface

The exposure of *E. coli* to individual AMPs or combinations thereof resulted in structural alterations to the bacterial cell surface. AFM imaging of untreated bacteria revealed regular rod-shaped cells, with numerous flagella and a cell surface covered in small granules and irregular flat grooves (Fig. 3). Exposure to 20 μM abaecin resulted in cells that appeared smoother than untreated controls, with less pronounced granules and grooves (Fig. 3). The changes were also observed in sectional profiles (Fig. 4). These data confirm our previous results [21,36]. Although cells incubated with 0.3 μM cecropin A retained their shape and flagella, they developed an additional flattened envelope. The topography of the cell surface also changed: the superficial granules became less sharply defined and more ovoid in morphology (Fig. 3) and new cavities appeared on the surface, 15–20 nm deep and 100–150 nm in diameter (Fig. 4). In contrast, the cells incubated with 0.05 μM stomoxyn underwent much more severe changes in morphology, losing their regular shape and envelope and developing larger cavities than the cells exposed to cecropin A, i.e. circular or longitudinal in profile, up to 40 nm deep and 150–200 nm wide (Fig. 4). The cell surface also became partially covered with patches of unidentified material, probably originating from the damaged cell envelope (Fig. 3).

The exposure of bacteria to the combinations of AMPs led to a distinct set of surface alterations compared to bacteria treated with individual peptides, but the precise nature of these alterations differed according to the mixture of AMPs applied (Figs. 3 and 4). In the cultures treated with 20 μM abaecin and 0.3 μM cecropin A, there was a substantial loss of surface granularity and the surface instead became covered in flat furrows, 2–4 nm deep and 50–80 nm wide (Fig. 4). These cells were also surrounded by a damaged, jagged and uneven cell envelope. In contrast, the bacteria treated with 20 μM abaecin and 0.05 μM stomoxyn developed a

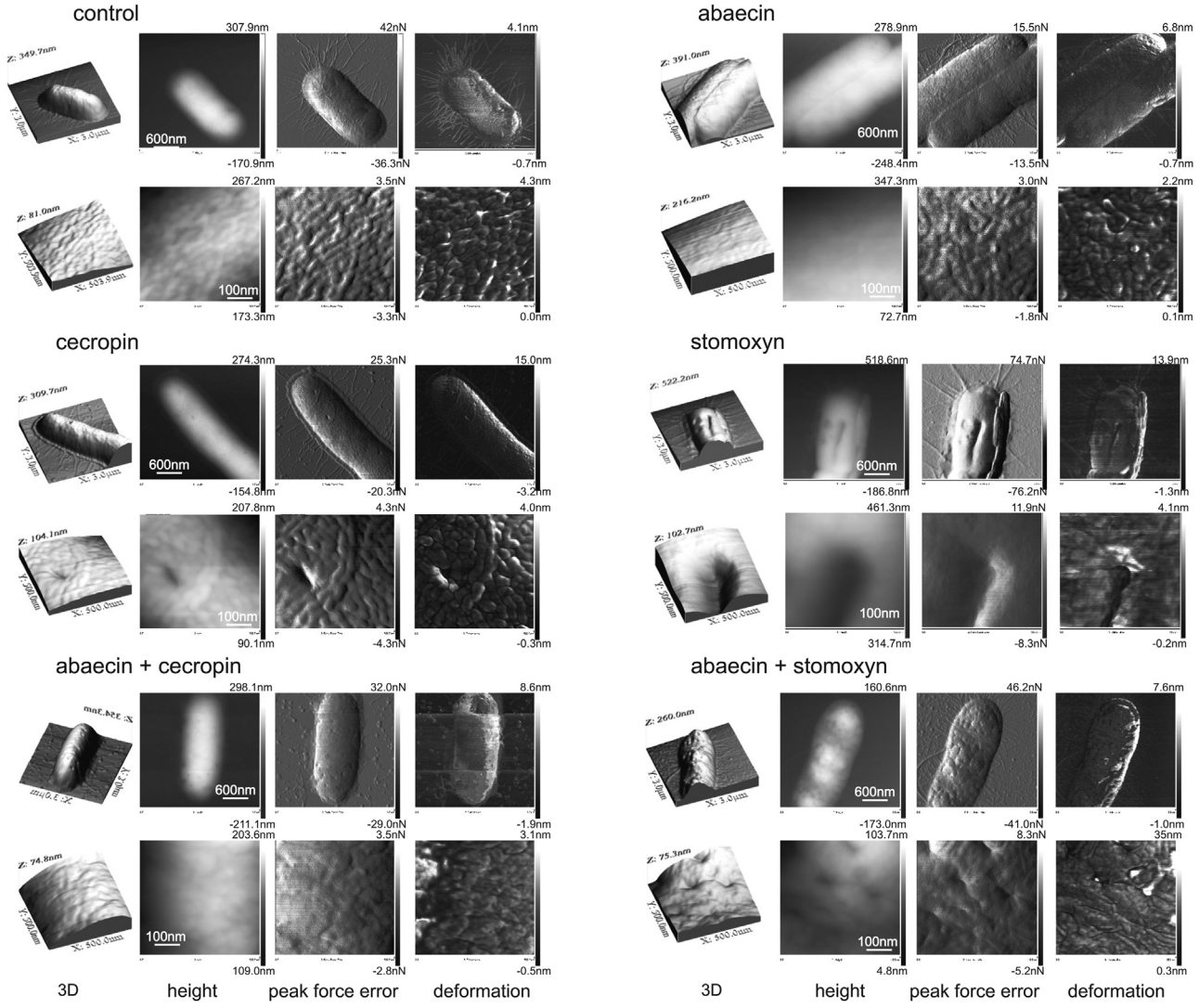

Fig. 3. Gross morphology of *E. coli* JM83 cells treated with 20 μM abaecin, 0.3 μM cecropin A, and 0.05 μM stomoxyn, alone or in combination. The control cells were incubated without AMPs. Scale bars in each left panel refer to the entire row.

Table 2
The effect of AMPs on the roughness of *E. coli* JM83 cells.

	Control	Cecropin A (0.3 μM)	Stomoxyn (0.05 μM)	Abaecin (20 μM)	Cecropin A + Abaecin	Stomoxyn + Abaecin
Roughness RMS value (nm) ± SD	5.983[b] (±1.81)	7.369[c] (±2.149)	9.700[a] (±4.031)	5.558[b] (±2.423)	7.214[c] (±3.001)	7.318[c] (±1.625)

Statistical significance between treatments: $p < 0.05$ (ANOVA; Fisher's LSD test). Values marked with the same letter are not significantly different.

more uneven surface, with irregular recesses and furrows 5–10 nm deep and 50–100 nm wide (Fig. 4). These substantial changes in surface topography were accompanied by significant differences in the cell surface RMS roughness compared to untreated controls, with the exception of cells treated with abaecin alone (Table 2).

4. Discussion

We have previously shown that bumblebee abaecin alone does not affect bacterial proliferation at concentrations of up to 200 μM and has negligible membrane permeabilization activity (~5%) at concentrations of up to 20 μM, but can potentiate activity of hymenoptaecin (a pore-forming peptide also from the bumblebee) and thus reduce the MIC of hymenoptaecin required for membrane permeabilization [21]. This interaction could be specific, given that the two AMPs are naturally co-expressed in response to a bacterial challenge, but could also represent a more general mechanism. We therefore tested the activity of abaecin with two other pore-forming AMPs: *H. cecropia* cecropin A and *S. calcitrans* stomoxyn [8,15]. Both are α-helical cysteine-free peptides, whereas abaecin is a proline-rich peptide [15,25].

We found that *E. coli* strain D31 showed no evidence of growth inhibition in the presence of up to 200 μM abaecin, in agreement with our previous studies [21]. In contrast, Rees et al. observed significant growth inhibition, although they used an abaecin fraction prepared by HPLC [25] and the precise concentration was not determined, so it is possible they applied a much higher concentration than we used in our experiments. They also used a different bacterial strain (*E. coli* strain D22) which has a membrane defect that might pre-dispose the bacteria to membrane permeabilization.

Stomoxyn and cecropin A have similar structures and their mechanisms of action are therefore related. Stomoxyn adopts a typ-

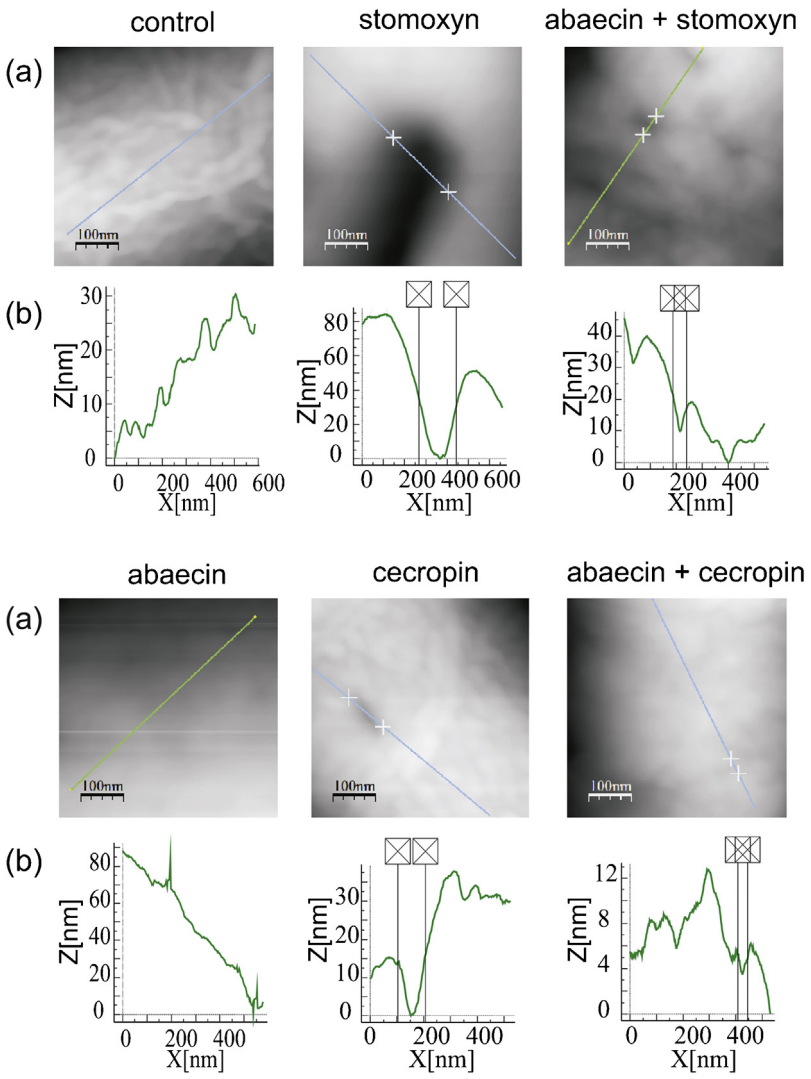

Fig. 4. Surface profiles of *E. coli* JM83 cells treated with 20 μM abaecin, 0.3 μM cecropin A, and 0.05 μM stomoxyn, alone or in combination. The control cells were incubated without AMPs. In each panel, (a) shows height images of the bacterial cell surface and (b) shows section profiles corresponding to the lines marked in (a).

ical cecropin structure, including cationic N-terminal amphipathic and C-terminal hydrophobic α-helices, but the C-terminal helix of stomoxyn is not well defined. Both peptides are thought to form transmembrane pores via a "carpet-like" mechanism [15]. These peptides must interact with outer membrane components initially in order to reach the inner membrane of Gram-negative bacteria, which is the target for most cationic defense peptides. The cationic peptides are likely to bind negatively-charged lipopolysaccharides thus displacing the divalent ions that stabilize the outer membrane structure [7,10,34].

Our AFM data revealed that the application of each AMP individually in sublethal concentrations caused a unique spectrum of structural changes on the bacterial cell surface, reflecting the initial interactions described above (Figs. 3 and 4). The structural differences are likely to reflect the different properties and/or conformations of each peptide, which determine the envelope components with which they interact, the binding affinity and the impact on normal envelope functions. Even so, none of the interactions appeared to be detrimental because bacterial growth and proliferation were unaffected (Fig. 1).

Whereas sublethal concentrations of each individual AMP had no effect on bacterial growth, combinations of abaecin and each of the two pore-forming peptides resulted in profound changes to the cell surface (Figs. 3 and 4) as well as an absolute inhibition of bacterial proliferation (Fig. 1) suggesting that abaecin reduces the MIC of the other AMPs as previously observed for bumblebee hymenoptaecin [21]. The increase in permeabilization caused by the combined AMPs was quantified by measuring the β-galactosidase activity (Fig. 2) and was eventually lethal to the cells. Both heterologous peptides were therefore potentiated by abaecin in a similar way to hymenoptaecin. As a result, in the presence of sublethal concentrations of these pore-forming peptides, 20 μM abaecin was sufficient to inhibit bacterial growth and proliferation (Fig. 1). Considering the concentrations, stomoxyn is more potent than cecropin A in terms of potentiating the effect of abaecin, but both are more potent than hymenoptaecin [21]. Based on its structural characteristics, stomoxyn functions like cecropin A by disrupting bacterial membranes [15]. The differences in antimicrobial potency may also reflect their hydrophobicity: hydropathy plots (Fig. 5) show that the C-terminal α-helices of cecropin A and stomoxyn are much more hydrophobic than the short, scattered hydrophobic regions in glycine-rich hymenoptaecin, increasing their affinity for the bacterial membrane. This is also supported by the grand average hydropathicity (GRAVY) scores of hymenoptaecin (−0.859), cecropin A (−0.073) and stomoxyn (−0.002). Although hymenoptaecin is not as potent as stomoxyn

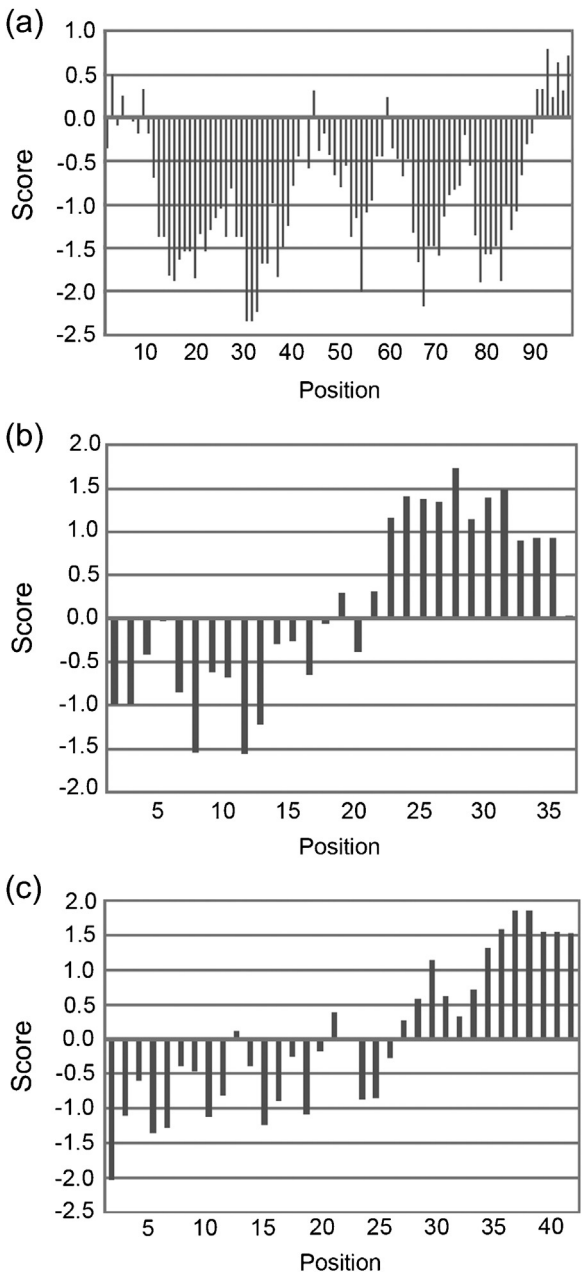

Fig. 5. Hydropathy plots of hymenoptaecin (a), cecropin A (b), and stomoxyn (c). Hydrophobic and hydrophilic domains are shown above and below zero, respectively. The size of the window is nine residues.

and cecropin A in terms of membrane permeabilization, it nevertheless fulfils this role in the bumblebee [21]. The AMP potentiation mechanism is likely to be based on reciprocal functional augmentation: the pore-forming peptides facilitate the uptake of abaecin which then binds to DnaK and inhibits its chaperone activity; this in turn accelerates the loss of membrane integrity by interrupting repair processes [21]. Similar phenomena have been observed with other combinations of peptides, such as LL-37 and human β-defensin [3,12,16,17,18,33,35].

Our data suggest that potentiating functional interactions between abaecin and pore-forming peptides are not restricted to AMPs produced in the same species and are likely to represent a more general mechanism that works across species barriers. Combinations of AMPs from different species could therefore be tested to determine whether this increases their potency and/or the range of pathogens that become susceptible, as already reported for cecropin + mellitin [1], attacin + thanatin [32] and cecropin + thanatin [13]. Such strategies may be used in future combination antimicrobial therapy, to confer pathogen resistance in transgenic plants [2], or to develop cosmetic products that deter dermatological pathogens [24].

5. Conclusions

The bumblebee long-chain proline-rich peptide abaecin can potentiate the activity of pore-forming AMPs from other species, in this case cecropin A and stomoxyn. This potentiating functional interaction represents a promising therapeutic scenario because the potency and range of combinations of AMPs is likely to extend well beyond the capabilities of individual peptides. In addition to combination therapy, individual synthetic peptides could be designed containing the active sequences of two or more natural AMPs, which could then be used for comprehensive clinical testing against diverse range of bacteria. This provides a real opportunity to address the rising threat of multidrug resistant pathogens that are recalcitrant to conventional antibiotics.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2016.01.016.

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