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Characterization of a chimeric antimicrobial peptide uncovers evolutionary significance of exon-shuffling

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ABSTRACT

The abaecin family comprises a class of proline-rich antimicrobial peptides (AMPs) with restricted distribution in hymenopteran insects. Intriguingly, in the parasitic wasp *Nasonia vitripennis* its members (termed nabaecin-1 to -3) have gained a carboxyl terminal glycine-rich antimicrobial unit through exon-shuffling. Here, we describe cDNA cloning of nabaecin-3 and the donor gene (*navitripenicin*) of the shuffling, and structural and functional features of nabaecin-3 and its two domains (respectively called amino-terminal abaecin unit (NtAU) and carboxyl-terminal navitripenicin unit (CtNU)). *Nabaecin-3* and *navitripenicin* were found to be transcriptionally up-regulated in response to bacterial challenge. By using recombinant expression and chemical synthesis techniques, we produced nabaecin-3, NtAU and CtNU. Circular dichroism (CD) analyses show that these peptides remarkably differ in their structures. Functionally, nabaecin-3 displayed a wide spectrum of antimicrobial activity against an array of bacteria, yeasts and fungi at micromolar concentrations, while CtNU only had a weak antibacterial activity and NtAU completely lacked activity. Our results indicate that in *Nasonia* the antimicrobial function of abaecin depends on the combination of NtAU with CtNU and thus suggest a new role of exon-shuffling in buffering loss-of-function mutation of a gene.

1. Introduction

Antimicrobial peptides (AMPs) are typically cationic and often composed of less than 100 amino acids, which constitute key components of the innate immune system of multicellular organisms [1]. Based on diverse primary and secondary structures, these molecules can be divided into three distinct groups: (1) linear AMPs with an α-helical conformation, such as melittin from bee venom [2], meucins from scorpion venom [3] and magainins from frog skin [4]; (2) disulfide bridge-containing AMPs with different structural scaffolds. Typical examples include a variety of defensins from plants to fungi and animals [5,6]; (3) peptides rich in single types of amino acids, such as proline-rich PR-39 from pig neutrophil [7] and glycine-rich hymenoptaecin from bee venom [8].

Abaecin is a proline-rich AMP with moderate but equal activity against both Gram-negative and Gram-positive bacteria [9]. Since its first discovery in the hemolymph of *Apis mellifera*, some abaecin-like peptides were also found in other hymenopteran insects, such as *Apis cerana*, *Bombus ignitus*, *Bombus terrestris*, *Pteromalus puparum* and the parasitoid *Nasonia vitripennis* [10]. More recently, Zhang and Zhu employed computational approaches to mine genomes of seven ant species and identified nine new abaecin-like proteins that can be distinguished into two distinct groups [11]. Mature abaecins are composed of 31–39 amino acids with a consensus motif P-X(2,3)-PGXGP(F/Y)NP(K/R/H) (X, any amino acids). In the *N. vitripennis* genome, *abaecin* appears to have undergone exon-shuffling and gene duplication to generate a multigene family of three members (nabaecin-1 to -3), each having an extended carboxyl-terminus acquired from a paralogous ancestor of *navitripenicin*, a predicted *N. vitripennis* gene encoding a putative glycine-rich AMP [10]. Nabaecins are synthesized as a precursor composed of a hydrophobic signal peptide followed by a mature peptide comprising the amino-terminal abaecin unit (NtAU) corresponding to *A. mellifera* abaecin and the carboxyl-terminal navitripenicin unit (CtNU). This chimeric structure was considered as a consequence of exon-shuffling [10]. The restricted phylogenetic distribution of the abaecin family indicates that it was originated from a common ancestor of hymenopteran insects and nabaecins emerged when wasps appeared (Fig. 1).

In this work, we firstly confirmed our previous genomic predictions on *nabaecin-3* and its donor gene – *navitripenicin* [10] by RT-PCR and revealed their inducible expression features in response to bacterial infection. Subsequently, we recombinantly expressed nabaecin-3 and CtNU in an *Escherichia coli* system and chemically synthesized NtAU. Structural and functional studies

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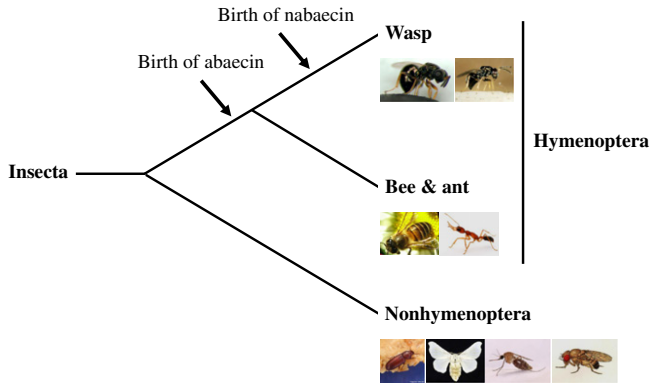

Fig. 1. The evolution of the abaecin family in hymenoptera. Single-domain of abaecins represents the ancestor form of two-domains of nabaecins in the wasp lineage.

uncovered that the antimicrobial activity of nabaecin-3 depends on a combination of NtAU with CtNU.

2. Materials and methods

2.1. cDNA cloning and semi-quantitative RT-PCR

Preparation of total RNAs and the first-strand cDNAs of non-infected and infected *N. vitripennis* adults were performed according to the methods described previously [10,12]. The first-strand cDNAs were used as template for standard nested PCR, in which primer pairs – nabaecin-1/3/3AP or navitripenicin-FP/3AP were used for the first amplification of *nabaecin-3* or *navitripenicin*; and nabaecin-3 FP/nabaecin-3 RP or navitri-FN/3AP for their second amplification. Nucleotide sequences of nabaecin-3 and navitripenicin have been confirmed by DNA sequencing and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/) under accession numbers of JF935449 and JN626959, respectively. Primers used in this study are all listed in Table 1.

Semi-quantitative RT-PCR was used to detect the inducible expression of *nabaecin-3* and *navitripenicin* before and after bacterial infection. PCR was conducted with initial denaturation at 94 °C for 5 min followed by different cycles (i.e. 25, 30, and 35) of denaturing (94 °C) for 45 s, annealing (62 °C) for 45 s and extension (72 °C) for 1 min, and final extension at 72 °C for 5 min. 5 μl of PCR products were analyzed and visualized by electrophoresis in a 1% agarose gel containing ethidium bromide. The ribosome protein Rp49 gene was used as an internal control [10].

2.2. Expression, purification and characterization of recombinant proteins

The amplified gene was inserted into pET-28a at *Eco*R I and *Sal* I sites with an enterokinase (EK) cleavage site (DDDDK) at the 5′-end of the coding region for the removal of the carrier. pET-28a-nabaecin-3 and pET-28a-CtNU were transformed into *E. coli* BL21(DE3)-plysS cells for protein expression.

Methods for the expression and purification of the two proteins have been described previously [13]. Lyophilized fusion proteins were digested in 20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 2 mM $CaCl_2$ by EK (SinoBio, Shanghai) at room temperature for 3 h. Recombinant products were finally purified by RP-HPLC.

Molecular weights (MWs) of peptides were determined by MALDI-TOF mass spectra on a Kratos PC Axima CFR plus (Shimadzu Co. Ltd., Kyoto).

2.3. Chemical synthesis of peptides

A peptide corresponding to NtAU was synthesized by Xi'an Huachen Bio-Technology Co., Ltd. (Xian, China) and its purity was confirmed by RP-HPLC and MALDI-TOF.

2.4. CD spectroscopy

CD spectra of nabaecin-3, NtAU and CtNU were recorded on a JASCO J-720 spectropolarimeter (Jasco, Tokyo, Japan) at a protein concentration of 0.3 mg/ml dissolved in water. Spectra were measured at 20 °C from 250 to 190 nm by using a quartz cell of 1.0 mm thickness. The CD spectra measure was performed by averaging three scans. Data are expressed as molar circular dichroism ($\delta\varepsilon$).

2.5. Antimicrobial assays

Methods for evaluating antimicrobial activity of nabaecin-3, NtAU and CtNU have been described previously [6].

2.6. Sequence analysis

For pairs of genes, numbers of synonymous (*Ks*) and nonsynonymous substitutions (*Ka*) per site were estimated by K-Estimator 6.1 [14].

3. Results and discussion

3.1. Molecular cloning and inducible expression of nabaecin-3 and navitripenicin

Nabaecin-3 and *navitripenicin* are two newly predicted genes based on their genomic sequences, in which a conserved phase-0 intron between the receptor (an orthologous ancestor of bee and ant abaecins) and the donor (a paralogous ancestor of *navitripenicin*) is crucial evidence for exon-shuffling to generate the chimeric structure of nabaecin-3 [10]. However, such prediction lacks experimental support. By using RT-PCR, we isolated and sequenced cDNA clones of nabaecin-3 and navitripenicin, which confirmed our previous prediction and indicate the presence of the intron (Fig. 2A). Because the intron is located at both ends of the mature peptide of abaecin and of the propeptide of navitripenicin, the shuffled product thus includes the signal peptide

Table 1
PCR primers used in this study.

Name	Sequences (5′ to 3′)	Usage
Nabaecin-1/3*	ATGAAGTTCCTMGCMAGTTT	RT-PCR
Navitri-FP	ATGAAGGCATTCGCTCTGA	RT-PCR
Navitri-FN	TTGGTGGCATTTTCATCG	RT-PCR
Nabaecin-3 FP	GAATTCGATGACGATGACAAGTACGTACCAAAGATTCC	Construction of recombinant plasmid
Nabaecin-3 RP	ATGTCGACTTATTTTCCCCAGCTAAAG	Construction of recombinant plasmid
CtNU FP	TGAATTCGATGACGATGACAAGTCTCCCAAAGACAATGGA	Construction of recombinant plasmid

* Degenerate primer: M = A or C.

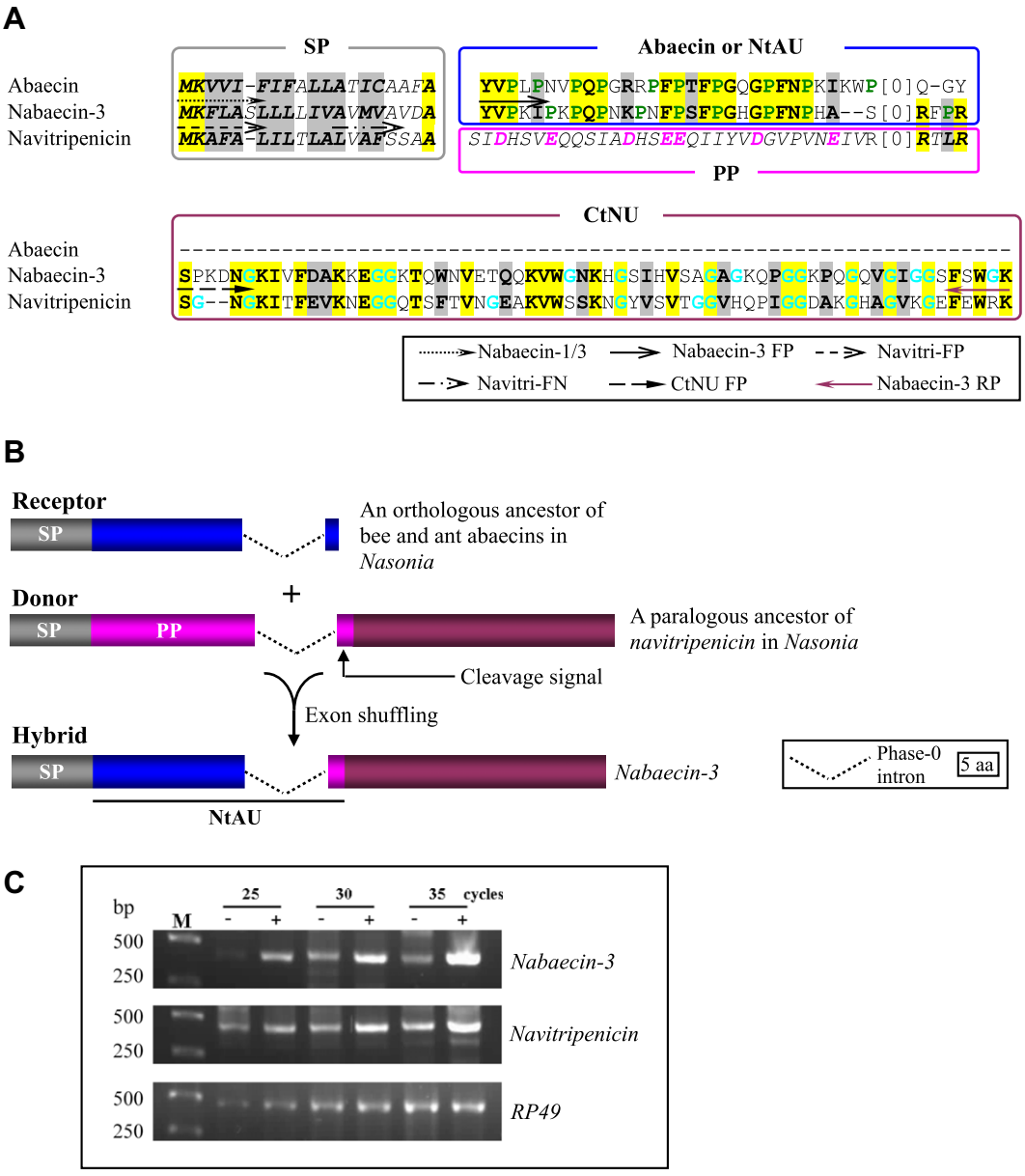

Fig. 2. Molecular evidence for the birth of nabaecin-3 by exon-shuffling. (A) Molecular cloning confirms the presence of one conserved phase-0 intron between nabaecin-3 and navitripenicin. Primers are indicated by arrows. Identical sites are shadowed in yellow and conservation replacements in grey. Different domains in the precursors of abaecin, nabaecin-3 and navitripenicin are boxed in different colors. Prolines in abaecin and NtAU and glycines in navitripenicin and CtNU are highlighted in green and cyan, respectively. SP, signal peptide, and PP, propeptide. Acidic amino acids in the propeptide of navitripenicin are highlighted in pink; (B) exon-shuffling between an orthologous ancestor of bee and ant abaecins (receptor) and a paralogous ancestor of *navitripenicin* (donor) [10]; (C) semi-quantitative RT-PCR detecting the expression level alteration of *nabaecin-3* and *navitripenicin* in response to bacterial infection. RP49 was used as an internal control. M: DNA marker. −: non-challenged. +: challenged. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and nearly complete mature peptide of abaecin and the entire mature navitripenicin as well as its propeptide cleavage signal (Fig. 2B).

Subsequently, we detected the alteration of *nabaecin-3* and *navitripenicin* at the transcriptional level by semi-quantitative RT-PCR. Results show that they both were able to be amplified from non-infected *N. vitripennis* adults (Fig. 2C). However, the transcripts of these two genes were up-regulated after bacterial challenge, suggesting that they are likely involved in the immune defense of *N. vitripennis*. In fact, transcriptional up-regulation of AMPs is a common defense strategy in rapidly clearing microbial infection [15]. For example, AMPs in *B. terrestris*, such as *abaecin*, *defensin* and *hymenoptaecin*, are induced by bacterial challenge [16].

3.2. Production of recombinant and synthetic peptides

To evaluate functional significance of exon-shuffling, we produced recombinant nabaecin-3 and CtNU (residues 34–98) as well as synthetic NtAU (residues 1–33) for structural and functional analyses. The two recombinant peptides were expressed as His-tagged fusion proteins in soluble form. After EK digestion, nabaecin-3 and CtNU were further purified by RP-HPLC, where the digested products of nabaecin-3 fusion proteins formed two main peaks, respectively eluted at retention time (T_R) of 21.5 min and 22 min (Fig. 3A). Their MWs identified by MALDI-TOF are 7484.8 Da and 10542.1 Da, matching theoretical values of 7482.5 Da for residues 1–66 and of 10538.9 Da for the full-length peptide, indicating that the former represents a nonspecific

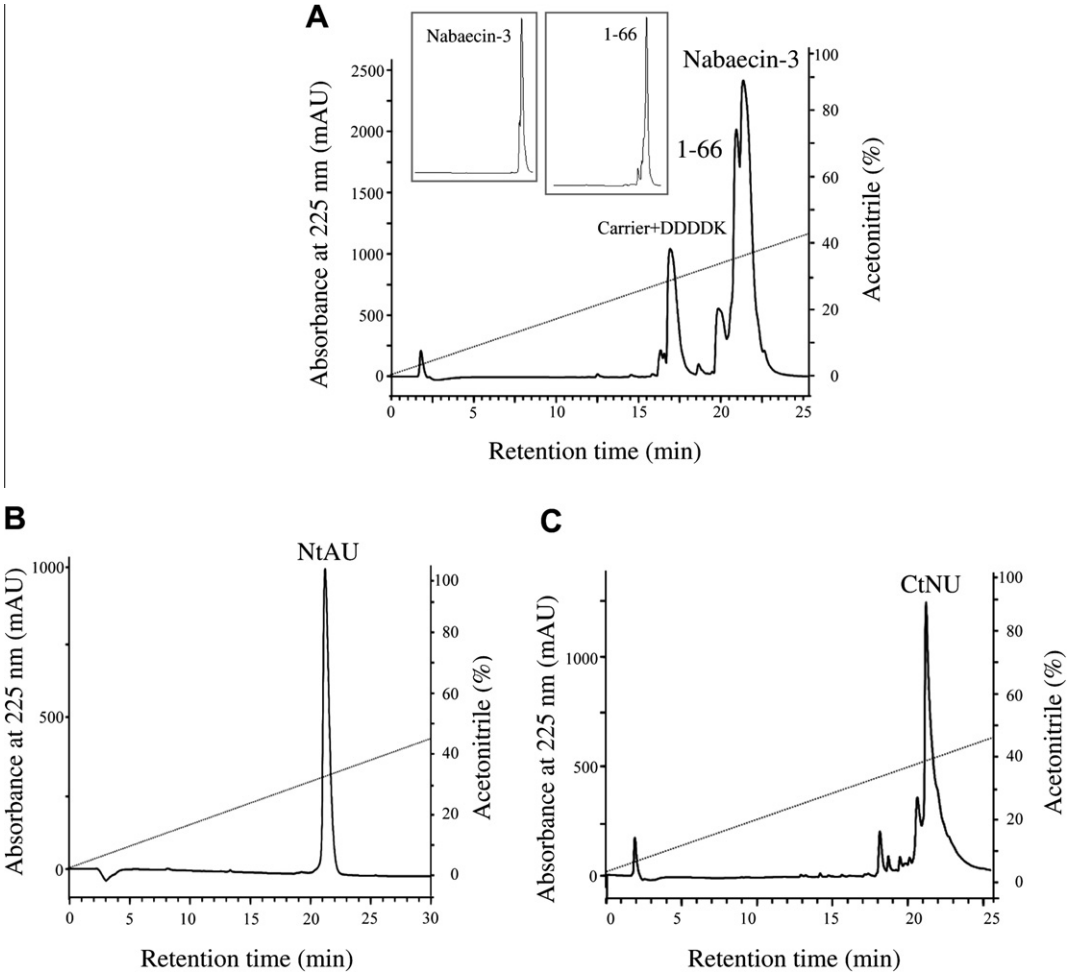

Fig. 3. RP-HPLC showing purification of nabaecin-3, NtAU and CtNU. C18 column was equilibrated with 0.1% TFA and purified proteins were eluted from the column with a linear gradient from 0% to 60% acetonitrile in 0.1% TFA within 40 min. Insets: re-purified recombinant abaecin-3 and its truncated product (residues 1–66).

cleavage product of nabaecin-3 composed of the entire NtAU and part of CtNU. Given the non-specific cleavage occurring between K66 and R67, it is likely due to trace amounts of trypsin contamination in EK. The recombinant CtNU eluted at T_R of 21.2 min as a single peak (Fig. 3B) and had a MW of 6803.5, consistent with the calculated value of 6802.6 Da. The final yields of nabaecin-3 and CtNU are each about 4 mg/l and 3 mg/l *E. coli* culture. Synthetic NtAU was also purified by RP-HPLC (Fig. 3C) and eluted at 21.5 min with an experimental MW of 3754.7 Da, perfectly matching the calculated MW of 3754.3 Da.

3.3. Structural and functional features of nabaecin-3, NtAU and CtNU

We analyzed secondary structures of nabaecin-3, NtAU and CtNU by CD. As shown in Fig. 4, the CD spectrum of nabaecin-3 displays a strong minimum around 200 nm, indicative of random coli structures. Interestingly, the CD spectrum of NtAU had a positive maximum around 190 nm and a negative minimum at 210 nm, suggesting the presence of some secondary structural elements, whereas CtNU seems to form an ordered α-helical structure due to a positive maximum around 200 nm and negative minimum at 210 nm and 230 nm.

Antimicrobial activities of nabaecin-3, NtAU and CtNU were evaluated against an array of different microbial strains (Table 2). Results show that nabaecin-3 exhibited a wide-spectrum of toxicity on Gram-negative, Gram-positive bacteria, fungi and yeasts at micromolar concentrations, in which *Bacillus megaterium* is the most sensitive species with a C_L value of 0.63 μM. On the contrary, CtNU only inhibited the growth of two Gram-negative strains (*Stenotrophomonas sp.* YC-1 and *E. coli*) and one Gram-positive bacterium (*B. megaterium*) with >10-fold lower potency than nabaecin-3. NtAU lacked any activity on all the strains used here. These results indicate that antimicrobial function of nabaecin-3 depends on the combination of NtAU with CtNU.

3.4. Evolutionary significance of exon-shuffling

In this work, we systemically compared antimicrobial activities of nabaecin-3 and its two separate domains (NtAU and CtNU). In comparison with the highly active full-length molecule, NtAU displayed no effect on all the microbial organisms and CtNU only had weak activity. These observations indicate that although nabaecin-3 has a putative cleavage signal (RFPR) (Fig. 2A) originated from the propeptide of navitripenicin, the abaecin unit needs CtNU to work as an entire functional element rather than processed as two separate products. In addition, although NtAU shares >50% sequence similarity with *A. mellifera* abaecin, it lacked any antimicrobial activity. In fact, previous studies have shown that for soluble enzymes approximately 40% of mutations reduce or completely abolish the activity of the mutated protein [17]. It is thus possible that such loss in NtAU is due to the effect of deleterious mutations during evolution, as evidenced by the *Ka*/*Ks* value [18] (<1)

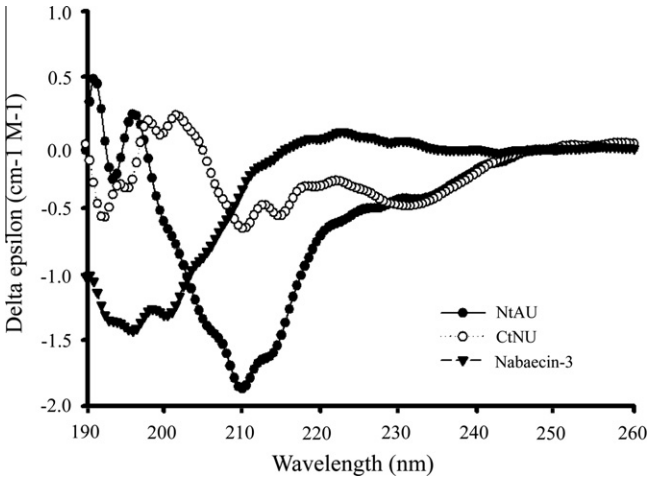

Fig. 4. Circular dichroism (CD) spectra of nabaecin-3, NtAU and CtNU, which were measured at a peptide concentration of 0.3 mg/ml in water from 190 to 250 nm. $\Delta\varepsilon$ corresponds to the variation of molar amino acid residue absorption expressed in $M^{-1}\ cm^{-1}$.

Table 2
Lethal concentrations (C_L) of nabaecin-3, NtNU and CtNU against various microorganisms.

	C_L (μM)		
	Nabaecin-3	NtNU	CtNU
Gram-negative bacteria			
*Stenotrophomonas sp.*YC-1	0.75	N.A.	3.91
Escherichia.coli ATCC 25922	0.92	N.A.	10.21
Salmonella.typhimurium CCTCC AB 94007	7.59	N.A.	N.A.
Serratia marcescens CCTCC AB 90025	20.33	N.A.	N.A.
Gram-positive bacteria			
Bacillus megaterium CCTCC AB 91020	0.63	N.A.	5.68
Fungi			
Geotrichum.candidum CCTCC AY 93038	2.20	N.A.	N.A.
Trichophyton rubrum	4.82	N.A.	N.A.
Aspergillus fumigatus CCTCC AF 93024	22.19	N.A.	N.A.
Neurospora crassa	10.32	N.A.	N.A.
Candida albicans	10.81	N.A.	N.A.

N.A.: no activity, indicating that no inhibition zone was observed at 1.0 nmol peptide each well.

between NtAU and abaecin. In this case, evolutionary gain of CtNU by exon-shuffling could help retain the activity of abaecin gene in *Nasonia*. Given significant structural difference between nabaecin-3 and its two domains, we speculate that the combination of the two domains might trigger a conformation facilitating the formation of a surface for antimicrobial activity.

Exon-shuffling, initially proposed by Walter Gilbert in 1978, has been proved to be a major molecular evolutionary mechanism to create new genes with novel functions [19,20]. In this work, we provide molecular evidence to support initial donor-recipient relationship of the exon-shuffling between a paralogous ancestor of *navitripenicin* and an orthologous ancestor of bee and ant abaecins [10]. Importantly, the present work for the first time suggests that exon-shuffling is not only a vehicle of functional divergence but also a means to buffer deleterious effects of mutations in an immune molecule. A similar role has also been proposed for gene duplication to avoid deleterious effects of mutations [21]. Given that proline-rich and glycine-rich AMPs are two classes of nature-occurring effectors, our work could be valuable in guiding the design of new-type chimeric molecules with higher microbial potency.

Acknowledgments

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