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NTNH protein: more than a bodyguard for botulinum neurotoxins

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Abstract

As one of the most fatal substances, botulinum neurotoxins (BoNTs) have never acted solo to accomplish their formidable missions. Most notably, non-toxic non-hemagglutinin (NTNH), a protein co-secreted with BoNT by bacteria, plays critical roles to stabilize and protect BoNT by tightly associating with it to form the minimal progenitor toxin complex (M-PTC). A new cryo-EM structure of the M-PTC of a BoNT-like toxin from *Weissella oryzae* (BoNT/Wo) reveals similar assembly modes between M-PTC/Wo and that of other BoNTs, yet also reveals some unique structural features of NTNH/Wo. These findings shed new light on the potential versatile roles of NTNH during BoNT intoxication.

Graphical Abstract

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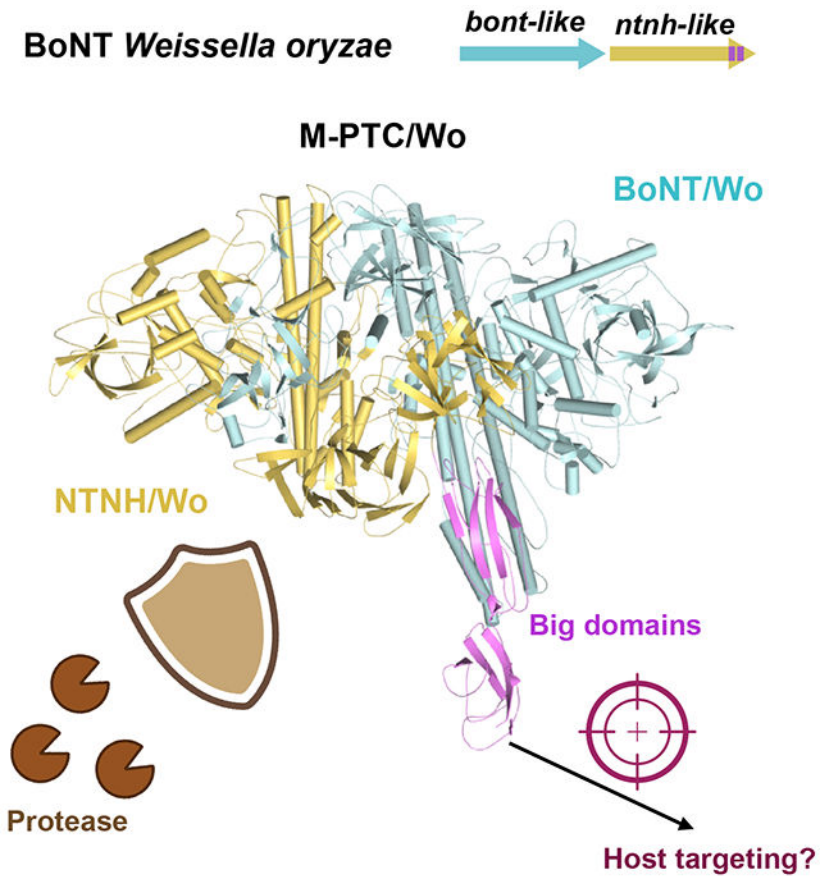
Author contributions

L.G. made Figure 1. L.G. and R.J. conducted the writing.

Conflicts of interest

The authors declare no conflict of interest.



NTNH is a protein co-secreted with BoNT by bacteria. A new cryo-EM structure of a BoNT-like toxin from *Weissella oryzae* (BoNT/Wo) in complex with its NTNH suggests that NTNH/Wo plays a conserved role as canonical NTNHs in stabilizing and protecting BoNTs. Furthermore, NTNH/Wo displays some unique structural features including two extra Big domains, suggesting NTNH/Wo may be involved in host targeting and play additional roles during BoNT intoxication.

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Keywords

botulinum neurotoxin; botulism; non-toxic non-hemagglutinin; progenitor toxin complex

Introduction

Botulinum neurotoxins (BoNTs) stand as some of the most lethal toxins causing the severe neuroparalytic disease, botulism. These toxins are produced predominantly by *Clostridium botulinum* and a few related species [1]. Historically, BoNTs are divided into seven major antigenic types (serotypes A-G) based upon neutralization with specific antiserum. These toxins are encoded in one of two major neurotoxin gene clusters (NGCs) in BoNT-producing bacteria including the hemagglutinin *ha* gene cluster and the *orfX* gene cluster [2]. Both NGCs carry the BoNT-encoding gene (*bont*) and a gene (*ntnh*) encoding a protein

termed non-toxic non-hemagglutinin (NTNH), while the *ha* cluster contains three additional hemagglutinin genes (*ha17*, *ha33*, and *ha70*) and the *orfX* cluster carries a different set of genes including *orfX1*, *orfX2*, *orfX3*, and *p47* (Figure 1A). These non-BoNT proteins are collectively termed neurotoxin-associated proteins (NAPs).

BoNTs are naturally produced together with NAPs in the form of progenitor toxin complexes (PTCs). A common feature among all BoNTs and BoNT-like toxins known to date is that BoNT assembles with its corresponding NTNH into a ~300 kDa minimal PTC (M-PTC) [3–5]. As revealed by the well-studied M-PTC/A, BoNT/A and NTNH/A form an inter-locked complex burying a large solvent-accessible area, which serves to mutually protect each other in order to survive the harsh environment (e.g., low pH and protease-rich) of patient's gastrointestinal (GI) tract [3, 4, 6]. Remarkably, the assembly of the M-PTC is delicately regulated by the environmental pH, which allows a timely release of BoNT upon transitioning from the acidic GI to the relatively safe systemic circulation where a bodyguard is no longer needed [3, 7].

For the HA-type PTCs, the M-PTC can further associate with HA17, HA33, and HA70 to form a large PTC (L-PTC) that adopts an Apollo lunar module-like architecture with the M-PTC mimicking the "ascent stage" and the HA proteins forming a three-arm "descent stage" [8]. The HA complex is believed to facilitate toxin absorption across the intestinal barrier via cell surface carbohydrates and a host adhesion protein E-cadherin [6, 9]. Interestingly, even though the M-PTC structure of the toxins encoded in the *orfX* NGC highly resembles that of the HA type toxins [5], no sequence nor structural similarity was observed between OrfX/P47 and HA proteins. The physiological relevance of these OrfX/P47 proteins to BoNT function remains elusive.

Thanks to the fast development of high-throughput sequencing techniques and bioinformatics for genomic data-mining, several novel BoNTs have been identified recently, including BoNT/HA and BoNT/X that are encoded in the genomes of *Clostridium botulinum*, as well as at least three distantly related BoNT-like toxins such as BoNT/En from *Enterococcus faecium*, PMP1 from *Paraclostridium bifermentans*, and BoNT/Wo from *Weissella oryzae (Wo)* [10–12]. As expected, most of these newly identified toxins are encoded in either the *ha* or the *orfX* NGC. But surprisingly, the NGC of BoNT/Wo-producing bacterium only contains *bont-ntnh* genes. Furthermore, a prior bioinformatics study revealed that NTNH/Wo contains two unique bacterial immunoglobulin-like (Big) domains, suggesting NTNH/Wo may pick up new "skills" during evolution (Figure 1A) [12].

Cryo-EM structure: The minimal progenitor toxin complex of BoNT/Wo

In this issue of *The FEBS Journal*, Kosenina and colleagues reported the cryo-EM structure of the M-PTC of BoNT/Wo [13]. The structure reveals that BoNT/Wo and NTNH/Wo form an interlocked compact complex and the overall architecture is highly similar to the crystal structures of the M-PTCs of BoNT/A and BoNT/E that are encoded in the *ha* and *orfX* NGC, respectively (Figure 1B, 1C) [3, 5]. Moreover, M-PTC/Wo also displays an M-PTC/A-like pH-dependent association: BoNT/Wo and NTNH/Wo assemble with each other at acidic conditions, but fall apart at neutral or basic pH. These common features

shared between M-PTC/Wo and that of other BoNTs suggest that BoNT/Wo likely needs NTNH/Wo as a bodyguard in its natural environment, although its biological functions and potential host targets remain unknown.

Detailed structural analyses reveal several unique features of BoNT/Wo and NTNH/Wo that may give us a glimpse of their functions. For example, BoNT/Wo has a unique configuration in the catalytic pocket on its light chain (LC); it does not have a ganglioside-binding pocket nor any known protein-receptor binding motif; and it lacks a disulfide bond bridging the LC and the heavy chain (HC) that is indispensable in all other BoNTs. The most striking observation is two tandem Big domains extending downstream of the heavy chain (nHC) of NTNH/Wo (Figure 1B). This confirms the previous bioinformatics analysis of BoNT/Wo NGC [12]. Interestingly, the homologous Big domains have been identified in several bacterial cell surface proteins that are involved in host cell adhesion, invasion, and protein-protein interactions in general [14]. It thus raises an intriguing question that these Big domains on NTNH/Wo may facilitate BoNT/Wo to fulfill its host interaction as no *ha* or *orfX* genes were identified in *W. oryzae* genome. Investigating the role of Big domains and host targets of BoNT/Wo should be an exciting topic for future research.

Conclusions and Perspectives

Now that we have seen representative M-PTC structures from each of the three unique BoNT NGCs, it is clear that all M-PTCs adopt a tightly bound interlocked complex and display a dynamic pH-dependent assembly, suggesting NTNHs play a conserved role in stabilizing and protecting BoNTs in their potentially widely different native environments (Figure 1C). Nevertheless, NTNHs seem to pick up custom-made “skills” to accommodate the needs of their corresponding BoNTs. For example, NTNH/A has a short ~40 amino acid loop (termed nLoop) inserted in its nLC that helps to attach M-PTC/A to the HA complex [3, 4, 6, 8]. The nLoop is conserved in all the HA-type NTNHs, suggesting these BoNTs likely rely on their NTNHs to coordinate the protection and delivery components of the PTC. In contrast, none of the OrfX-type NTNHs have nLoop, which is consistent with the lack of HA proteins. But then come the questions of whether and how NTNH in the *orfX* NGC work with OrfX proteins and/or P47 during intoxication, which remain a major challenge to address. In the case of NTNH/Wo, two Big domains are inserted into its nH_C domain while its nH_N domain is noticeably shorter than that of other NTNHs (Figure 1C), which sheds new light on the potential new functions it may have ‘picked up’ to support BoNT/Wo. Taken together, these findings suggest that NTNHs may provide an evolutionarily flexible structural platform for diverse BoNT variants to adapt to their unique environments and attack specific host organisms and tissues. Thus, teaching NTNH new tricks appears to align favorably with the evolutionary progress of BoNTs and BoNT-like toxins, and the revelation of additional roles of NTNHs awaits further exploration in future studies.

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Abbreviations

BoNT	botulinum neurotoxin
HA	hemagglutinin
NTNH	non-toxic non-hemagglutinin
NGC	neurotoxin gene cluster
NAP	neurotoxin-associated protein
M-PTC	minimal progenitor toxin complex
L-PTC	large progenitor toxin complex
Big domain	bacterial immunoglobulin-like domain
LC	light chain
HC	heavy chain
nHC	heavy chain of NTNH
Wo	Weissella oryzae

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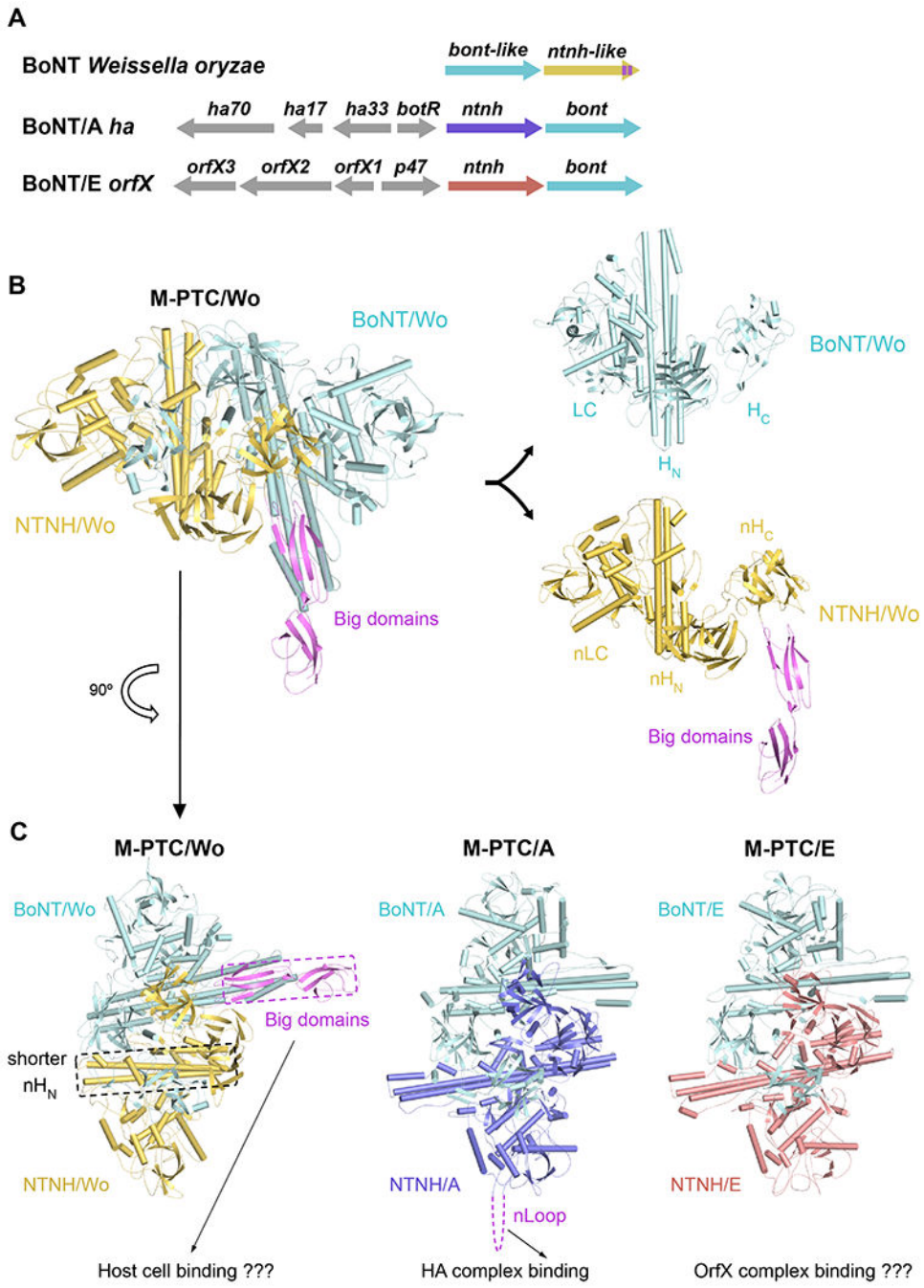

Figure 1. Structures of the M-PTC from three different BoNT neurotoxin gene clusters.

(A) Composition of the *ha*, *orfX*, and *Weissella oryzae* neurotoxin gene clusters. (B) Ribbon and cylinder representations of the BoNT/Wo–NTNH/Wo complex. BoNT/Wo is colored in pale blue and NTNH/Wo is colored in yellow orange with the Big domains colored in violet. (C) Structural comparison of M-PTC/Wo, M-PTC/A (PDB: 3V0A), and M-PTC/E (PDB: 4ZKT). The structure figures were prepared with PyMOL (Schrödinger Inc.).