Botulinum neurotoxins exploit host digestive proteases to boost their oral toxicity via activating OrfX/P47

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Botulinum neurotoxins (BoNTs), produced by *Clostridium botulinum* and related species, are among the most potent natural toxins known and are responsible for botulism, a rare but potentially fatal paralytic disease. Beyond their pathogenic role, BoNTs are also valuable therapeutic agents, underscoring the importance of understanding their molecular mechanisms of action. BoNTs are encoded within specialised neurotoxin gene clusters that include *bont*, *ntnh* (encoding nontoxic non-hemagglutinin), and accessory genes, arranged in either *ha*-type or *orfX/p47*-type clusters. While the haemagglutinins (HAs) have established functions in enhancing oral toxicity, the role of the OrfX and P47 proteins has remained elusive.

In this study, we investigated the physiological function of the OrfX1, OrfX2, OrfX3, and P47 proteins encoded by the *orfX/p47* cluster. Using mouse bioassays, we demonstrate that the combined action of all four components markedly enhances the oral toxicity of BoNTs, a process dependent on proteolytic activation by host digestive enzymes encountered during intoxication. Notably, OrfX2 adopts a self-inhibiting conformation and engages with BoNT via NTNH only after protease activation. Cryo-electron microscopy reveals that two protease-activated OrfX2 molecules bind simultaneously to NTNH, forming a structural assembly critical for potentiation of BoNT oral toxicity.

Our comparative genomic and phylogenetic analyses revealed that *orfX/p47* clusters are conserved across diverse bacterial taxa and frequently co-occur with genes encoding other oral toxins, such as insecticidal crystal and vegetative insecticidal proteins. This widespread distribution suggests a broader, evolutionarily conserved role for the OrfX/P47 system in mediating oral toxicity.

The findings reveal a previously unrecognised mechanism by which BoNTs, and potentially other bacterial toxins, exploit host digestive proteases to activate a modular protein complex that enhances toxin stability or uptake. By elucidating the structural and functional interplay among BoNT, NTNH, and the OrfX/P47 system, this study offers new insights into toxin pathogenesis and opens avenues for developing improved countermeasures and biotechnological tools.