Photorhabdus bacterial toxins as a candidate for insect pest bio-management: an update

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Abstract: Insect-parasitic nematodes belonging to the families Heterorhabditidae and Steinernematidae have developed symbiotic relationships with bacteria belonging to the genera *Photorhabdus* and *Xenorhabdus*, respectively. The nematode–bacteria complex can kill the host insect within 24-48 h via septicemia or toxaemia. Bacteria (which lives in the nematode gut) enter the host through its nematode partner, and the nematode relies on bacteria for nutrition from liquefied host tissue. The bacteria kill the insect by using its arsenal of toxins and secondary metabolites. These nematodes have long been utilized as insect biological control agents, but their short shelf life and demand for a certain temperature and moisture range for field performance have limited their commercial use. A number of protein toxins from distinct Indian strains of *Photorhabdus* spp. were identified in our laboratory. The biological activity of candidate toxins was tested in *Galleria mellonella*, a model insect, and then in agriculturally important insects such as *Heliocoverpa armigera*, *Spodoptera litura*, *S. exigua* and *S. frugiperda*. When toxins was administered orally either via artificial diet or force-feeding, the toxins had shown catalytic activity on the insect gut epithelial cells and moved to hemocoel by proteolytically cleaving the basement membrane lining in the gut-hemocoel barrier. Following that, a cytotoxic effect on immunocytes or hemocytes was seen, similar to apoptosis or cell death. Toxins also had an immunomodulatory effect as documented by the elevated phenoloxidase activity in the hemolymph. These gut-active toxins putatively interact with different insect midgut receptors. Currently, we are pursuing RNAi knockdown of receptors to establish their role in disease development. The novel insecticidal toxins characterized from *Photorhabdus* spp. may provide a greater diversity of biotoxins at disposal for pest management either via transgenic mean or bio-protectant formulations.

Key words: Txp40; TcaB; insecticidal toxin; entomopathogenic nematode; insect gut receptor

The explosion of world's human population (an exponential increase from initial estimate of 0.35 billion during 14thcentury to current 7.9 billion that may reach up to 9 billion by 2050) has put an unrealistic demand on global crop production especially because of the shrinking arable land and a push towards water-intensive agriculture due to water scarcity. In parallel, insect pests consume approximately 20% of global agricultural yield. This problem is aggravated due to certain factors such as global climate change, cultivation of high input-requiring plant varieties and indiscriminate usage of chemical insecticides (Shankhu *et al*., 2020; Dutta *et al*., 2021a). An increased regulation of synthetic pesticides towards deregistration and restricted use is occurring frequently because of some alarming issues like pesticide resistance development in insects, adverse effect of insecticides on the environment and soil health, greater half-life of common insecticides in soil and non-

specific selectivity of insecticides in target organism with only handful of molecular targets including acetylcholinesterase (AchE) enzyme, voltage-gated sodium (Na_v) ion channels and glutamate-gated chlorine channel or Gamma-Aminobutyric Acid (GABA). Consequently, current growers are facing shortage of active insecticides amid the increasing incidence of newer insect pests.

For the control of insect pests in agriculture, alternative tactics such as the production of transgenic crops producing insecticidal protein have been used to great success. Today, the most successful protein toxins used in the development of transgenic crops are those produced by the bacterium *Bacillus thuringiensis*, which produces an insecticidal crystalline protein known as Bt delta endotoxin (also known as Cry toxin) that kills insects (Bravo *et al*., 2011, 2015). As a result of the widespread use of Bt-based biopesticides on a broad scale, as well as the commercial cultivation of transgenic plants expressing toxin genes, resistant insect populations are being emerged in countries such as India, China, and the United States (Tabashnik, 2015; Tabashnik and Carrière, 2017; Carrière *et al*., 2019). Thus, development of novel protein toxins is necessary in order to increase the diversity of genes available for pest control applications.

Biocontrol agents against insect pests, such as Entomopathogenic nematodes (EPNs), have been utilized for quite long, and the bacteria that they carry have been investigated in greater depth (Clarke, 2020). There are two bacterial genera, i.e. *Xenorhabdus* and *Photorhabdus*, which live in close association with nematode genera, *Steinernema* and *Heterorhabditis*, respectively, as symbiotic partners. Numerous insects, such as members of Coleoptera, Lepidoptera, Diptera, and Dictyoptera order can be infected and killed by this nematode-bacterium duo (Lacey *et al*., 2015; Garcia-del-Pino *et al*., 2018).

The symbiotic bacteria are harboured in the intestine of nematode infective juveniles (IJs), which are free-living stages. IJs scavenge for insect hosts in the soil and enter the hemocoel of the host through natural orifices or by penetrating the cuticle. The worms then liberate their symbiotic bacteria that proliferate rapidly and eventually kill the host insect within 24 to 48 hours. To complete development, worms feed on bacteria and dead insect tissues. After 2-3 generations, when the insect cadaver is deprived of nutrients and nematode densities approach critical levels, IJs harbouring their symbiotic bacteria emerge from the cadaver in quest of fresh host insects. These bacteria are quite fascinating in the bacterial world because they form mutualistic associations with one host, nematodes, and a harmful association with another phylum, insects (Clarke, 2020). A schematic representation of life cycle of an EPN (*Heterorhabditis indica* that harbors the bacterium *Photorhabdus akhurstii*) in a model insect, *Galleria mellonella* is given in Fig. 1.

The motile gram-negative bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. are member of Morganellaceae family that belong to Gammaproteobacteria class (Machado *et al*., 2018). *Xenorhabdus* spp.and *Photorhabdus* spp. cannot be directly used as bio-pesticides because of the bacterium's inability to survive in soil for longer period. In view of this, alternatively, insecticidal

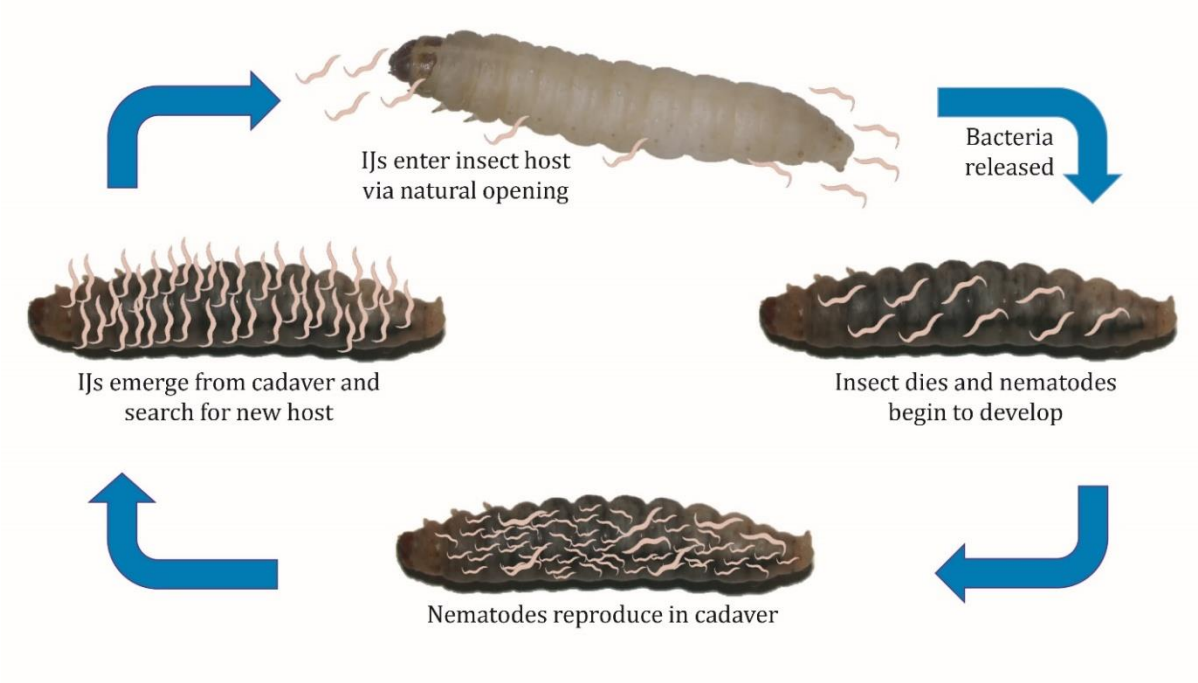

Fig. 1. Life cycle of EPN. IJs infect insects via an opening such as the anus, mouth, or spiracles, or by rupturing the cuticle. Once within the host, IJs shed their outer cuticle and begin consuming hemolymph that results in the regurgitation of symbionts. By 24-48 hours, the nematode–bacterium duo kill the host via septicemia or toxaemia. Next, the developing nematodes ingest the bacteria and bacterially metabolized liquefied insect tissues; nematodes mate together, reproduce and complete one or more generations before food resources get depleted. Bacteria re-associate with IJs that emerge from the depleted insect carcass in quest of new hosts.

property (specifically the toxin proteins) of these bacteria can be reproduced in other organisms such as bacteria or plant. In this direction, *Xenorhabdus* spp. and *Photorhabdus* encoded toxin genes and their secondary metabolites were studied by expressing those characters in heterologous hosts. For example, candidate toxins isolated from *Photorhabdus luminescens* when cloned and expressed in another bacterium, *Escherichia coli*, toxins displayed unprecedented pesticidal potential (Bowen *et al*., 1998). Subsequently, Morgan *et al*. (2001) reported the discovery of toxins from *Xenorhabdus nematophilus* and expressed its protein product in *E. coli* that conferred greater insecticidal property in the caterpillars of cabbage butterfly, *Pieris brassicae*. Since then a number of toxin genes were reported from both the *Xenorhabdus* spp. and *Photorhabdus* spp.

The whole genome sequencing of *Photorhabdus luminescens* strain TT01 and of *P. luminescens* strain W14exhibited that these bacteria constitute a wide spectrum of potential virulence factors such as high molecular weight toxin complexes (Tc), antibiotics, lipopolysaccharides, enzymes like proteases and lipases (ffrench-Constant *et al*., 2000; Duchaud *et al*., 2003). The Tc genes belong to a large gene family that harbour Tca, Tcb, Tcc and Tcd loci, with each locus exhibiting lethal effect on insect survival (Bowen *et al*., 1998; Blackburn *et al*., 1998; Waterfield *et al*., 2001). Other than that, various insecticidal toxins like Makes Caterpillar Floppy (Mcf) genes, *Photorhabdus* insect related (Pir) proteins,

Photorhabdus Virulence Cassettes (PVCs), Photox, Pit, Txp40 etc. have showed oral and injectable toxicity against a wide spectrum of insect pests (Daborn *et al*., 2002; Duchaud *et al*., 2003; Brown *et al*., 2006; Yang *et al*., 2006). Waterfield *et al*., (2005) proposed that for full functional activity of Tc toxin, different Tc components (TcdA-, TcdB- and TccC-like) must complement each other. However, the catalytic activity or mode of action of these candidate toxins may vary in different strains of *Photorhabdus* spp. Additionally, India as a vast country constitutes diverse and numerous agro-ecological zones and accordingly insect pest incidence substantially differs. Therefore, exploring the toxin gene diversity from the Indian strains of *Photorhabdus* spp. can make an arsenal of choices to prepare the nation to combat global food scarcity.

Role of bacterial toxins in insect management:

Tc gene homologues were characterized in different bacterial genera such as *Xenorhabdus* spp., *Yersinia* spp. (YenTc in *Yersinia entomophaga* and *Y. pestis*), *Burkholderia* spp., *Pseudomonas* spp., *Serratia entomophila* etc. The genomic structure of Tc locus has also been compared with the *Salmonella* plasmid-borne virulence factor loci, spvA and spvB (ffrench-Constant *et al*., 2007). Nevertheless, the utility of these bacterial toxins is yet to be realized in insect pest management. On the contrary, Cry toxins have been extensively used in world agriculture. Given its enormous volume of research globally, current section depicts the utility of Cry toxins in pest management in a detailed manner in the following paragraphs.

Bacillus thuringiensis is a gram-positive, spore forming, rod-shaped soil bacterium ubiquitously found from a vast range of ecosystems and niches like soil, water, dead insects and plant tissues, insect-feeding mammals, and even from the human tissues having severe necrosis (Höfte and Whiteley 1989; Raymond *et al*., 2010; Palma *et al*., 2014). The bacterium was first discovered by a Japanese sericulture specialist Ishiwatari Shigetane from the infected silkworms, which he named as *Bacillus sotto*. Later, Ernst Berliner (1911) rediscovered the bacterium as a causal organism of *Schlaffsucht* disease of Mediterranean flour moth larvae in Thuringia state of Germany. The molecular phylogenetic analyses showed the bacterium as a close relative to *Bacillus anthracis* and *B. cereus* (Guinebretière *et al*., 2013). *Bacillus thuringiensis* (Bt) is uniquely known for producing insecticidal proteins that are active against different insect Orders and also some species from other animal Phyla (Table 1). During the sporulation phase, the Bt strains produce crystal (Cry) proteins called as delta-endotoxins (Palma *et al*. 2014). The toxin protein crystals get solubilized upon reaching the insect midgut *per os*, and disrupt the midgut cells being proteolytically activated. The toxins then interact with the specified receptors localized onto midgut cell surface leading to pore formation in the cellular membrane resulting in insect death (Badran *et al*., 2016).

The Cry proteins produced by the Bt bacteria are especially toxic against the Hexapod Orders Lepidoptera, Diptera, Coleoptera and Hymenoptera (Bravo *et al*., 2007). The proteins collectively comprise about 50 subgroups with 200 members;

Table 1. A summarization of Bt derived Cry and Cyt endotoxins affecting different animal hosts (Palma *et al*., 2014)

Protein components	**Active against**
Cry51A, Cry32A, Cry22A, Cry15A, Cry9A-9C,9E, Cry8D,Cry7B, Cry2A, Cry1A-1K,	Lepidopteran insects
Cry49A,Cry48A, Cry47A,Cry44A, Cry39A, Cry32B-32D, Cry27A,Cry24C, Cry20A, Cry19A-19B, Cry16A, Cry11A-11B, Cry10, Cry4A-4B, Cry2A, Cry1A-1C, Cyt2A-2B, Cyt1A-1B,	Dipteran insects
Cry55A, Cry43A-43B, Cry37A, Cry36A, Cry35A-35B, Cry34A-34B, Cry23A, Cry22A-22B, Cry18A, Cry14A, Cry9D, Cry8A-8G, Cry7A, Cry3A-3C, Cyt2C, Cyt1A-1B	Coleopteran insects
Cry22A, Cry5A, Cry3A	Hymenopteran insects
Cry11A, Cry3A, Cry2A	Hemipteran insects
Cry55A, Cry21A, Cry14A, Cry13A, Cry12A, Cry6A-6B, Cry5A-5B	Nematodes
Cry1Ab	Gastropods

and analyses of tertiary structures by X-ray crystallography suggested their structural similarity that aid in similar mode of action for all of them (Bravo *et al*., 2007; de Maagd *et al*., 2001). They contain a bundle of seven α-helices at the N-terminus (Domain I) where the hydrophobic central helix-α5 is encircled by six amphipathic helices responsible for pore formation and membrane insertion. Domain II is comprised of three numbers of anti-parallel β-sheets having exposed loops, and Domain III forms a β-sandwich. The exposed regions present in Domain II and Domain III primarily helps in receptor binding at the midgut membrane. More specifically, the Domain II of the Cry toxin shares structural similarity with carbohydrate binding proteins like vitelline, lectin jacalin, lectin Mpa; whereas Domain III shows similarity to the cellulose binding domains of β-glucoronidase, galactose oxidase, sialidase, 1,4-β-glucanase C, and carbohydrate binding domains of β-galactosidase and xylanase U (de Maagd *et al*., 2003). The crystal inclusions of the Cry proteins, upon being ingested, get dissolved in the alkaline midgut; thereafter the solubilized inactive protoxins yield 60-70 kDa active toxin proteins being cleaved by the proteases (Bravo *et al*., 2005). The activation of toxins involves enzymatic removal of specific stretch from the N-and C-termini (Bravo *et al*., 2007). The *cry* genes in most of the Bt strains encode for endotoxic CRY proteins that are located at plasmids. Additionally, Bt bacteria also produces cytosolic (Cyt) proteins that are insecticidal in action (Li *et al*., 1996). The Cyt proteins, unlike cry proteins, contain one α-β domain with two outer layers of α-helix hairpins wrapped around the β-sheet; and are structurally related to volvatoxin A2 toxin of the straw mushroom *Volvariella volvacea* (Palma *et al*., 2014). Bt strains have also been found to secrete some insecticidal proteins during vegetative developmental phaseinto the growing media (Schnepf *et al*., 1998; Estruch *et al*., 1996). The secreted proteins are mainly comprised of two classes as vegetative insecticidal protein (Vip) and secreted insecticidal protein (Sip) (Donovan *et al*., 2006; Estruch *et al*., 1996). The Vips and Sips contain specific signal peptides that are conserved,

and cleaved after being secreted from the bacterium.

Bt derived spores and insecticidal Cry proteins are widely used to control the insect pests, particularly of Order Lepidoptera. The first Bt product, named as Sporeine, was commercialized in 1938 (Sanchis, 2011). Subsequently, several commercial preparations have been developed. To date, approximately 95% of the commercialized microbial bioagents are comprised of the Bt products (Schünemann *et al*., 2014). The majority of crystalline Bt based formulations are produced from Bt var. kurstaki HD1 (Cry1 Aa/Ab/Ac; Cry2Aa); Bt var. kurstaki HD73 (Cry1Ac); Bt var. aizawai HD137 (Cry1Aa/B/Ca/Da); Bt var. San Diego and var. Tenebrionis (Cry3Aa); and Bt var. israelensis (Cry4A/B; Cry11A; Cyt1Aa). These products are successfully used against several pests of agriculturally important crops like banana, cotton, citrus, corn, potato, tobacco, vegetables, and pasture (Schünemann *et al*., 2014). Further, transgenic crops expressing the Bt toxins provide a lucrative tool for integrated pest management. Notably, genetically modified tobacco was developed by the Belgian company Plant Genetic Systems (now under Bayer Crop Sciences) in 1985 was the starting point of Bt transgenics where the plants contained delta-endotoxin by expression of *cry* genes. Later, transgenic potato, corn, cotton, soybean, brinjal etc. have been developed expressing the Bt toxins; but not all of them have been given permission for open field cultivation (Abbas *et al*., 2018). Although the genetically engineered Bt crops reduce the pesticide usage and considerably drive off the destructive insect pest damage, they suffer from human and environment related controversies. Nevertheless, cultivation of Bt crops like Bt corn and Bt cotton showed no significant impact on the beneficial soil flora and fauna or non-target organisms (Mendelshon *et al*., 2003). Contrastingly, Abbas *et al*. (2018) showed that the Bt corn Mon810 produces specific toxins that interfere with human cell viability. In this line, In 2001, the US Environmental Protection Agency (EPA) reassessed the toxicity status of registered Bt corn with Cry1F, Cry1Ab; Bt potato with Cry3A; and Bt cotton with Cry1Ac on the non-target organisms including human and mammals; but the results revealed instability of the Bt in presence of human digestive fluids in gut and were degraded within seven minutes (Mendelshon *et al*., 2003).

The use of Bt formulations are relatively harmless to the humans and non-targets that provide considerable target specificity towards various insect pests. However, Bt-based transgenic plants and bio-pesticides are subjected to ethical considerations and safety issues, though researchers advocate for their cultivation based on laboratory and field studies. In this line, considerable dietary risk assessments, toxicological studies, allergenicity and digestibility studies, ecological risk assessments and environmental persistence studies should be conducted for safe use of the Bt-based products.

Photorhabdus toxins: New player in the game:

Photorhabdus genus is considered as the 'Pandora's box' for the discovery of novel insecticidal toxins, which could further be exploited as biocontrol agents either via transgenic mean or biopesticide formulation. Proteins identified in *Photorhabdus luminescens* including Tc, Mcf, PVC, binary toxins Pir, Photox etc. were found to be

insecticidal when injected into the insect hemocoel. Only a few of them were found to be orally active. Additionally, a multitude of virulence factors were mined from the whole genome sequence of *P. luminescens* strains TT01 and W14.

a. Toxin complexes:

The Tc toxins are very large oligomeric tripartite toxins (collectively more than 1.4 MDa in molecular mass) that constitute three basic components such as A, B, and C. Putatively TcA functions as receptor binding molecule and toxin translocator, TcC confers the toxic enzyme activity, while TcB function as a linker between TcA and TcC components. Blackburn *et al*. (1998) reported the Tc toxin's high insecticidal nature against lepidopteran pest, *Manduca sexta*. Histopathology of Tca in *M. sexta* was also reported and Tc mode of action was very similar to other gut active toxins that cause blebbing of the midgut epithelium and eventually lysis of epithelium leading to gut leakiness.

Notably, the nomenclature of Tc genes is quite complex and very confusing. Tripartite Tc genes can be divided into three basic genetic elements, i.e. TcA, TcB and TcC types, that are located at four different loci or four pathogenic islands named as Tca, Tcb, Tcc and Tcd. Astonishingly, *P. luminescens* strain TT01 and W14 constitute a wide variety of Tc genes, i.e. 7 TcA- and TcC-type genes (Roderer and Raunser, 2019). Waterfield *et al*. (2001) showed that Tcs carry multiple copies of TcdA (encode tcdA1, tcdA2 and tcdA3) and TcdB (tcdB1 and tcdB2) genes which are homologous to each other. TcaA/B, TccA/B, TcbA and TcdA loci are quite identical to each other based on their encoded protein types, suggesting the predominance of Tc gene isoforms in *P. luminescens* genome (Sheets and Aktories, 2017; Fig. 2).

Albeit the ambiguity in Tc gene nomenclature, individual Tc gene components such as Tca, Tcb, Tcc and Tcd separately exhibited partial toxicity to different insects when heterologously expressed in *E. coli* (Morgan *et al*., 2001; Waterfield *et al*., 2005; Yang and Waterfield 2013). Bacterially expressed TcdA1 and TcdB1 (independently) conferred oral insecticidal activity when extracted from the supernatant of *E. coli* cells (Waterfield *et al*., 2001). Most importantly, Liu *et al*. (2003) attempted the transgenic or *in planta* expression of *Photorhabdus luminescens* TcdA1 gene in model plant *Arabidopsis thaliana*, that conferred insecticidal activity against tobacco hornworm *Manduca sexta* and southern corn rootworm *Diabrotica undecimpunctata*. A 63 kDa protein (named as Toxin B) isolated from *P. luminescens*strain W14 conferred oral insecticidal activity against *D. undecimpunctata* (Guo *et al*., 1999). Blackburn *et al*. (2005) demonstrated that Tca is an orally active toxin against the whitefly pest *Bemisia tabaci* and Colarado potato beetle *Leptinotarsa decemlineata*; the two important subunits of Tca, i.e. TcaAii and TcaAiii were revealed to be least toxic in nature. Based on this, they concluded that toxic activity of Tca is depends on itself and not on the other subunits such as TcaAii and TcaAiii. On the contrary, it has also been demonstrated that complete toxicity of Tc can be restored if its individual components are co-expressed together (Waterfield *et al*., 2001). The Tc toxin of *P. luminescens* carries its cytotoxic activity in the C-terminal domain of TcC that is hypervariable in nature. This cytotoxic domain is cleaved out from the Tc holotoxin

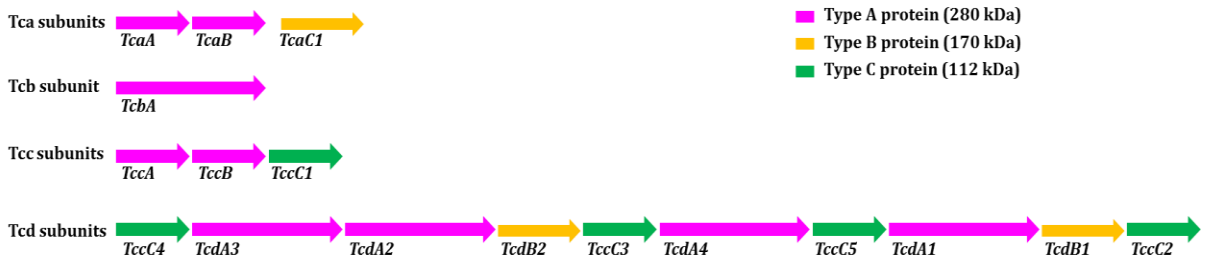

Fig. 2. The highly complicated nomenclature of Tc genes. Schematic representation of the structure and order of the *P. luminescens* Tc genes. Tc genes are found on four distinct pathogenic islands: Tca, Tcb, Tcc, and Tcd. The hue of a gene corresponds to the type of protein it encodes (Type A, B, or C). TcaA + TcaB are isoforms of the TccA + TccB locus and the TcdA or TcbA locus, respectively. Tc-binding and translocation are facilitated by Tca-like components. Tcb-related components act as a chaperone and connector between Tca and Tcc components. Tcc-like components contain the TccC3/C5 toxin enzyme. The TcaA/B, TccA/B, TcbA, and TcdA loci are nearly comparable in terms of the encoded protein type and sequence, indicating that Tc gene isoforms are prevalent in the *P. luminescens* genome.

(that comprises TcB-TcC complex) and liberated into the host cell cytosol during the Tc intoxication mechanism (Roderer *et al*., 2019).

A number of bacterial toxins target host actin molecules via ADP-ribosylation mechanism. Examples include binary toxins from *Clostridium botulinum* (C2 toxin), *C. perfringens* (iota toxin), *Clostridium difficile* transferase or CDT and vegetative insecticidal proteins (VIPs) from *Bacillus cereus*. *P. luminescens* TccC possesses ADP-ribosyltransferase (targets Rho proteins which are regulated by a GTPase cycle) activity and modifies actin molecule at Threonine-148 position. The translocation mechanism of Tc into target cells is assumed as injection syringe like phenomena, in which the toxic enzyme TccC5 causes actin polymerization that induces depolymerization of actin filaments and disintegration of actin cytoskeleton of the target host cells or insect hemocytes (Sheets and Aktories, 2017).

b. Makes Caterpillars Floppy (Mcf) toxins: Another important insecticidal toxin in *P. luminescens* genome is Mcf toxin. Mcf destroys the insect phagocytes, which are produced by insect immune reaction for killing the invading bacteria. Due to this, insect gut is badly damaged resulting in the floppy appearance of the larvae. Mcf protein mimics a BH3 domain that promotes apoptosis of the host mitochondrion. Mcf1-exposed cells exhibited fragmentation of host cell nucleus, host cell DNA laddering and activation of caspase-3 mechanism; all of that were indicative of cell death phenomena. Intriguingly, Mcf1 is detected in different genomic locations of different strains of *Photorhabdus* spp., implying that Mcf1 is a mobile genetic element in *Photorhabdus* genome. Conversely, Mcf1 is considered as an essential virulence factor as its homologues are found in all *Photorhabdus* strains, *Xenorhabdus* spp. and *Pseudomonas fluorescens* Pf-5 (Daborn *et al*., 2002; ffrench-Constant *et al*., 2007; ffrench-Constant and Dowling, 2014).

Waterfield *et al*. (2003) described a Mcf-like element in the genome of *P. luminescens* W14 strain and was named as Mcf2. Notwithstanding to Mcf1, amino termini of Mcf2 possesses a type III secretion system

delivered HrmA avirulence gene or effector of *Pseudomonas syringae*. The carboxyl termini of Mcf1 and Mcf2 possesses repeat in toxin (RTX)-like domains, which are known to be secreted by the type I secretion system. The presence of both type I and type III export machinery signature in Mcf genes is quite intriguing and provide some clue about how this large protein is secreted out of the bacterial cell.

c. Photorhabdus Virulence Cassettes (PVC):

The PVCs are another toxin-encoding islands (found as tandem repeats of prophage-like loci) prevalent in the *Photorhabdus* genome. PVC loci can move around and between the bacterial genomes, indicating its mobile nature. PVC locus constitutes a conserved PVC element (encodes phage components including phage tails and baseplates) and a payload region (encodes ORFs corresponding to bacterial effectors such as sepABC of *Serratia entomophila*). PVC has homology to *Serratia* anti-feeding prophage, which is found on the pADAP plasmid that utilizes a type IV DNA conjugation pilus, thus indicating a common mechanism of horizontal transfer of genetic elements between *Serratia* and *Photorhabdus*. Transmission electron microscopic (TEM) images of recombinant PVCs showed that PVCs contain a phage tail-like particle that is quite identical to R-type pyocins (bacteriocins may deliver the payload region encoded toxin into the host cell). *Photorhabdus* PVC effectors caused rearrangement of actin cytoskeleton in the cells of mammalian tissue culture suggesting its possible mode of action and led to reduction of circulating hemocytes in *G. mellonella* upon hemocoel injection (Yang *et al*. 2006; ffrench-Constant *et al*., 2007). Vlisidou *et al*. (2019) demonstrated that Pnf (a Rho-GTPase) is a PVC needle complex associated toxin, which disrupts the cytoskeleton through transglutamination. Using TEM and Western blot it was shown that Pnf is necessary for delivery of PVC component in the host via the cell membrane.

d. Photorhabdus insect related (Pir) binary toxins:

Waterfield *et al*. (2005) identified two ORFs, *plu4092* and *plu4436* were closely located to similar loci *plu4093* and *plu4437* within the *P. luminiscens* TT01 genome and showed oral toxicity against the adult and larvae of different mosquitoes including *Aedes aegypti*, *Anopheles gambiae* and *Culex pipiens*. Initially, these assumed to be orphan genes derived via gene duplication event were named later as '*Photorhabdus* insect related' (Pir) proteins A and B which are very similar to δ-endotoxins from Bt. Intriguingly, the presence of a number of enterobacterial repetitive intergenic consensus sequences around the Pir locus is suggestive of horizontal acquisition of *Pir* genes in *Photorhabdus* spp. Li *et al*. (2014) showed that PirAB protein is a binary protein that contains of pirA and pirB genes in separate genomic locations and were expressed together (with the help of a linker) in *E. coli*. The purified PirAB fusion and PirA and PirB mixture exhibited toxicity to the fourth-instar *S. exigua* caterpillarin terms of cell shrinkage, blebbing of cell membrane, condensation of nucleus and fragmented DNA. Abnormalities in the gut epithelium with more swelling and shedding of apical membranes in *Plutella xylostella* was observed when insects were ingested with recombinant *E. coli* co-expressing Pir

A and Pir B proteins (Blackburn *et al*., 2006). Ahantarig and coworkers (2009) showed that PirAB fusion protein confers greater toxicity compared to either of the individual PirA and PirB components or PirA/PirB mixture.

e. Photox toxin:

Visschedyk *et al*. (2010) reported a novel mART (mono-ADP-ribosyltransferase) enzyme produced by *P. luminescens* called Photox. It's a 46-kDa toxin and have shown similar homology in catalytic region of the actin-targeting mARTs. It also caused cytotoxic effect in yeasts. Similar to TccC5 mode of action, Photox causes polymerization of host cell actin cytoskeleton by modifying the actin molecule at arginine-177 position. Nevertheless, Photox translocation mechanism in target host cell is yet to be determined.

f. XaxAB binary toxin:

Zhang *et al*. (2014) analyzed the *P. luminescens* TT01 genomic sequence, during which they found *plu1961* and *plu1962* ORFs are similar to XaxAB binary toxin from *X. nematophila* that are involved in apoptosis and necrosis in the cell lines of insect and mammal. Vigneux *et al*. (2007) discovered that *Xenorhabdus* toxin α-xenorhabdolysin (Xax) causes apoptosis in both mammalian and insect cells by acting as a cytotoxin.

g. Photorhabdus insecticidal toxin (Pit):

A novel toxin Pit was cloned from *P. luminescens* subsp. *akhurstii* YNd185 and expressed in *E. coli* (Li *et al*., 2009); the purified Pit protein caused pronounced mortality in model insect *G. mellonella* (LD_{50} - 30 ng/larva) and economically important insect *S. litura* (LD_{50} – 191ng/larva).

h. Txp40:

Brown *et al*. (2006) identified a new 42 kDa protein toxin, Txp40 from *P. luminescens* strain V16. This toxin was ubiquitously present in other 59 strains of *Xenorhabdus* spp. and *Photorhabdus* spp. Txp40 cytotoxic effect was observed in different insects including *G. mellonella,* lepidopetran pests *Plodia interpunctella*, *Helicoverpa armigera* and the dipteran insect *Lucila cuprina* due to the oral feeding of recombinant protein. The primary site of action of Txp40 is insect gut epithelium and additionally causes some damage to fat bodies.

The potential of *Photorhabdus* toxin for pest management in Indian scenario:

In India, the amount of money spent on pesticides is estimated around annual US$650, which is mainly intended to control bollworm and sucking pest of cotton. Dhaliwal *et al*. (2015) recorded that Indian farming is suffering from a huge loss of US$36 billion (16.8% in terms of percentage of yield decline) annually owing to insect pest incidence. It has also been recorded that percentage of yield loss of crops because of insect pest attack in major crops rose from 7.2% to 23.3% during the period between pre-green revolution era (1960s) and post-green revolution era (2000s). Additionally, due to the adoption of Bt cotton in Indian agriculture (occupying more than 95% of cotton producing areas), the yield loss due to insect pest is estimated at around 30% (Dhaliwal *et al*., 2010, 2015).

A majority of previously reported toxins from *P. luminescens* is insecticidal when injected into the hemocoel of test insects,

and this is not desirable for further commercial deployment. In view of this, our laboratory is engaged in exploring the orally active toxins from Indian strains of *P. akhurstii* (a few of them recently elevated to novel Indian-subcontinent specific species and subspecies level such as *Photorhabdus hindustanensis* and *Photorhabdus akhurstii* subsp. *bharatensis*; Machado *et al*. 2021).

a. Txp40, PirA, TcaA, TcaB, TccC and TccC toxins from P. hindustanensis and P. akhurstii bharatensis:

Initially, a number of candidate genes were selected by *in silico* assay from the genome sequence database of *P. luminescens* strains TT01 and W14. A number of bacterial strains such as *P. hindustanensis*, *P. akhurstii bharatensis*, *P. akhurstii* strain IARI-SGGJ2 and *P. akhurstii* strain IARI-SGMS1 etc. were extracted from the IJs of *H. indica* nematode (collected from different geographical regions of India such as Meghalaya, Haryana, Gujarat and Maharashtra states) by culturing in nutrient bromothymol blue agar plates followed by 16S rDNA sequencing. The open reading frames (ORFs) or coding sequences of the toxin genes were cloned from different bacterial strains and transformed into *E. coli* cells. An extensive rapid virulence annotation (RVA) experiment was performed to screen out the potential virulence loci in these toxin candidates. For this, the recombinant *E. coli* clones containing toxin genes were intra-haemocoel injected (independently) into the fourth-instar larvae of model insect *Galleria mellonella*. Insect mortality data was recorded after 72 h of injection of six candidate toxins including Txp40, TcaA, TcaB, PirB, TccA and TccC. After identifying the potential ORFs that may correspond to bacterial virulence, these ORFs were cloned into a protein expression vector pET29a by restriction digestion method and subsequently transformed into heterologous host *E. coli* BL21(DE3) cells for functional investigation of these candidate toxins (Fig. 3).

Detailed *in silico* studies suggested the inter-strain gene sequence diversity at number of locations in the candidate toxins. This corroborated with the significant difference in the insecticidal activity of same protein toxins isolated from different bacterial strains, exemplifying the diverse gene pool of toxins in the Indian strains of *P. akhurstii* or *P. hindustanensis* leading to strain-dependent virulence in *Photorhabdus* bacterium (Dutta *et al*., 2020). It can be assumed that a single amino acid residue alteration might have significantly modified the biological activity conferred by the corresponding proteins. Notably, phylogenetic analysis of PirA showed its homologues in *P. akhurstii* and *P. luminescens* branch were nearer to a number of *Xenorhabdus* spp. such as *X. nematophila* and *X. poinarii*, but the same branched farthest from PirA homologues in *P. temperata*, *P. bodei*, *P. laumondii*, and *P. asymbiotica*. This exemplified the possibility of differential genomic location of PirA gene in several *Photorhabdus* species and thus, PirA is presumably mobile or transposable within the *Photorhabdus* genome (Dutta *et al*., 2020). TccC of *P. akhurstii* / *P. hindustanensis* contained YD repeat motifs, which are often located in the core domain of rearrangement hotspot (RHS) toxins, a part of polymorphic ABC toxins. Homologues of TccC were detected in 13 members of order Enterobacteriales including *P. luminescens*, *P. namnaonensis*, *P. khanii*, *P. temperata*, *P. thracensis*, *P.*

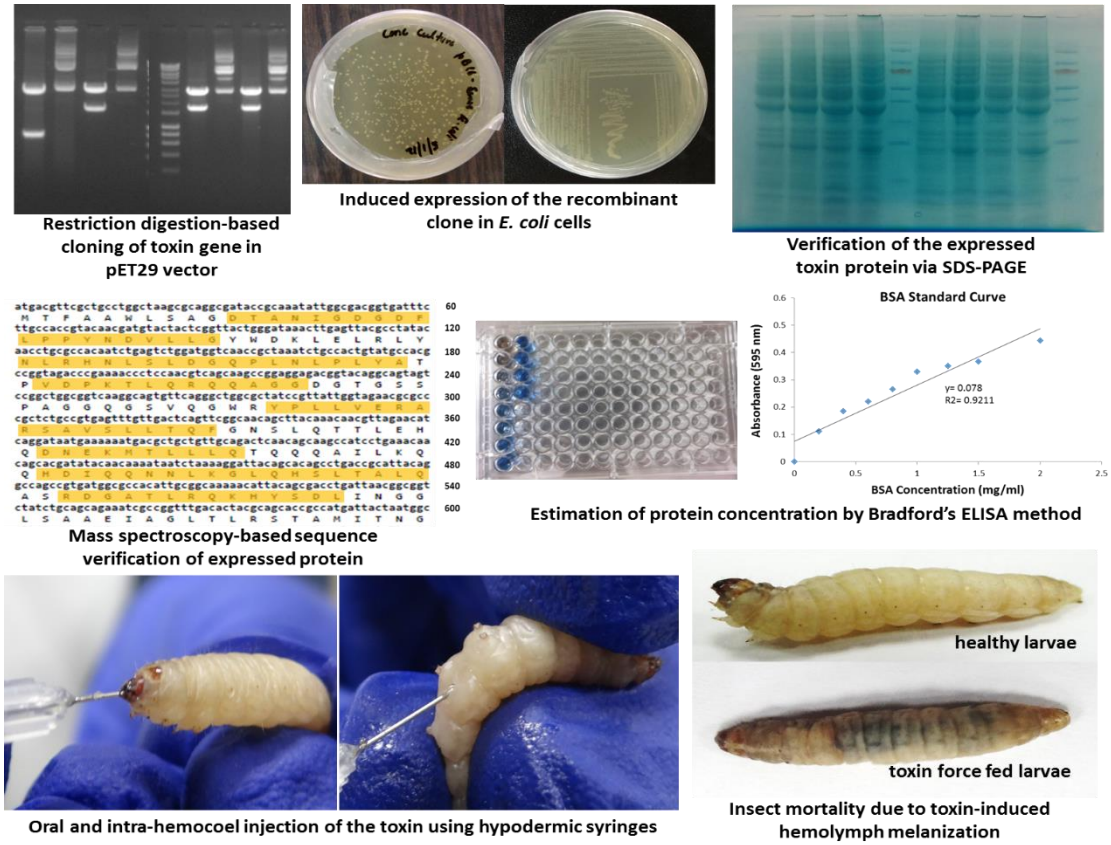

Fig. 3. Cloning, characterization and functional analysis of *Photorhabdus* toxins in *G. mellonella* fourth-instar larvae.

bodei, *P. laumondii*, *X. nematophila* and *X. bovienii*. On the contrary, TcaA of *P. akhurstii* / *P. hindustanensis* contained DNRLK/RE domain, which is predominantly found in *Bacillus* spp. Thus, TcaA homologues were detected in 16 members of Enterobacteriales order including a number of diverse genera such as *Yersinia*, *Erwinia*, *Burkholderia*, *Paraburkholderia*, *Aeromonas*, *Shewanella*, *Bacillus* and *Paenibacillus*. Similarly, TccA homologues were encountered in 15 members of Enterobacteriales order including *Burkholderia*, *Enterobacter*, *Salmonella* and *Granulibacter* (Dutta *et al*., 2020).

A *P. luminescens* Txp40 homologue was cloned from *P. hindustanensis*, *P. akhurstii bharatensis* and expressed in *E. coli* bacterium. The molecular weight of the purified, expressed protein was 37 kDa (encoded by an ORF of 1008 bp). The toxin protein conferred both injectable and oral toxicity in *G. mellonella* (Mathur *et al*., 2018). This was in stark contrast to original Txp40 protein (identified in *P. luminescens* strain V16) which was of 42 kDa in size and showed only injectable (no oral) insecticidal toxicity (Brown *et al*., 2006). Further, we demonstrated that Txp40 could confer both oral and injectable toxicity in agriculturally-significant insects such as cotton bollworm *Helicoverpa armigera*, tobacco cutworm *Spodoptera litura* and beet armyworm *S. exigua*. Haemocoel injection of the toxin in test insects (fourth-instar) resulted in the dose-dependent decline of total haemocytes (circulatory) and reduction in viability of haemocytes. Txp40 introduction in the hemolymph elevated the phenoloxidase (PO) activity to such extent that unrestricted

melanization occurred ultimately leading to larval death. TEM-based histopathology study revealed the extensive damage in midgut epithelium indicating the entry of toxin from haemocoel to gut through leaky septate junctions or catalytic action of the toxin molecule (Shankhu *et al*., 2020). A domain conservation study (that includes multiple threading and segment assembly technique) of TcaB revealed its potential homologues as a binary toxin of *Yersinia entomophila* (YaxAB), a binary toxin of *Xenorhabdus nematophila* (XaxAB), a cytotoxin of *Vibrio cholerae* (makA) and ADP-ribosyltransferase enzyme of *Legionella pneumophila* (Shankhu *et al*., 2020).

A C-terminal domain of TcaB toxin (1713 bp) was cloned from *P. akhurstii bharatensis*, *P. akhurstii* IARI-SGMS1 and expressed in heterologous host *E. coli*; the molecular weight of the purified, expressed protein was 63 kDa. TcaB toxin exhibited both injectable and oral toxicity in beehive pest *Galleria mellonella* (Mathur *et al*., 2019). Upon hemocoel injection, TcaB conferred pronounced cytotoxicity and immunomodulatory activity (in terms of increased phenoloxidase activity in the haemolymph). Hemocytes displayed typical morphological aberrations including cell shrinkage, blebbing of cell membrane, nuclear condensation and degradation, ultimately leading to apoptotic cell death (Mathur *et al*., 2019). TcaB constitutes several functional domains including a zinc metalloprotease / metazincin domain (42-170 aa; HEXXH signature) that is the determining factor of bacterial virulence (indicative of TcaB catalytic activity determinant), a transcription regulator motif (108-178 aa) overlapping the metazincin domain and a receptor binding domain (283-565 aa). TcaB domain conservation analysis showed its notable homologues as TcdA1 toxin of *P. luminescens* (Protein Data Bank accession number: 1VW1), Tc toxin of *Yersinia entomophaga* (6OGD), Cry2A toxin of *B. thuringinesis* (1I5P) and a crystal protein of *Bacillus* sp. (1J0M). The oral delivery of TcaB caused extensive damage in the gut epithelium of *G. mellonella* fourth-instar larvae during 6 to 24 h of incubation period; a gradual decline in gut homeostasis led to movement of TcaB into the hemocoel. Consequently, upon migration from alimentary canal to body cavity via leaky gut, TcaB conferred an unprecedented cytotoxicity and immunostimulatory activity in the *G. mellonella* haemocoel after 6 to 24 h of oral delivery (Santhoshkumar *et al*., 2021). The gut-active nature of the TcaB toxin was also demonstrated in the agriculturally-important insects including fall armyworm *Spodoptera frugiperda*, *S. litura* and *H. armigera* (Dutta *et al*., 2021a; Fig. 4).

b. Galtox from P. hindustanensis and P. akhurstii bharatensis:

During the search of potential Photox toxin variants in the genomes of seven *Photorhabdus* symbionts from *Heterorhabditis* nematodes of Indian subcontinent, a novel protein toxin Galtox (35 kDa in molecular weight) was identified serendipitously. Unlike of Photox (46 kDa toxin), Galtox did not confer oral toxicity in *G. mellonella* and do not encode the characteristic actin-targeting mono-ADP-ribosyltransferase (mART) domain. Galtox conferred rapid insecticidal activity via intra-hemocoel injection with LD_{50} values of 3.1-31.2 ng per g of *G. mellonella* body mass. Domain conservation analysis indicated the homology of Galtox with other

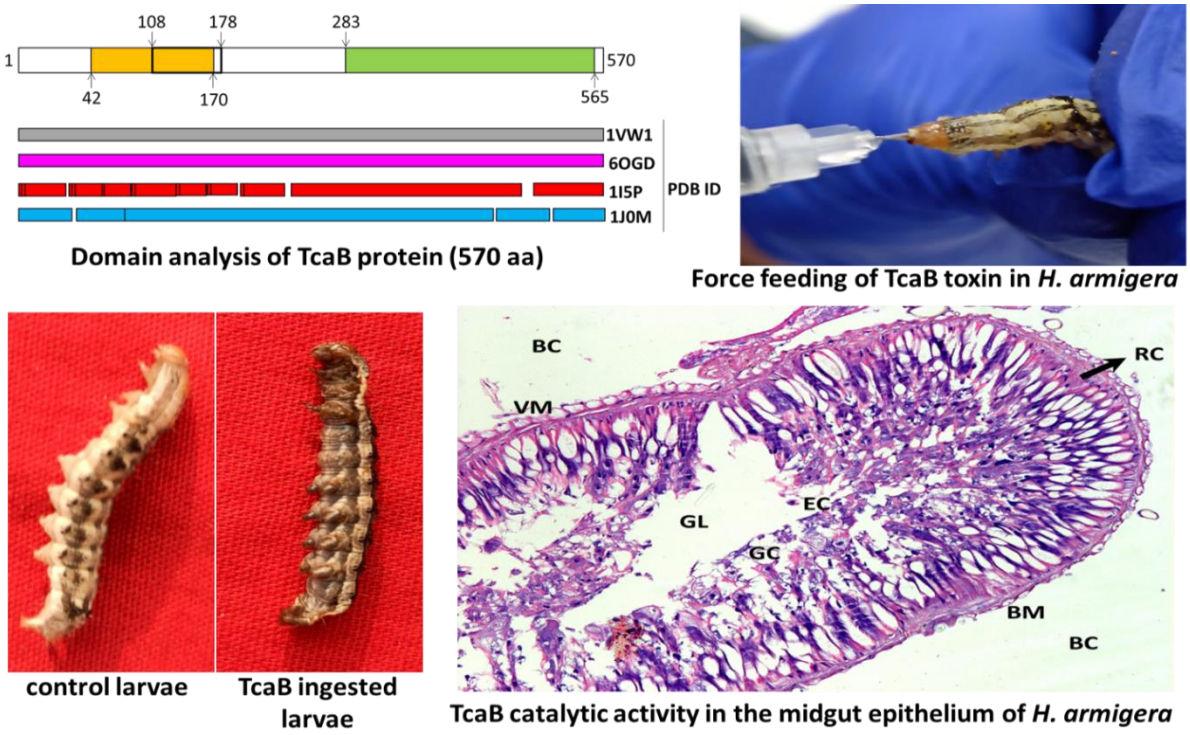

Fig. 4. TcaB induced gut leakiness in *H. armigera*. TcaB contains a catalytic domain (indicated in yellow box), a transcriptional regulator motif (empty box) overlapping the catalytic domain and a receptor binding domain (green box). TcaB conserved domains are homologous to a number of bacterial toxins (PDB accession numbers: 1VW1, 6OGD 1I5P and 1J0M). Upon oral admistration of TcaB in the fourth-instar larvae, TcaB caused degeneration of epithelial cells (EC), goblet cells (GC) and regenerative cells (RC) which sloughed off into the gut lumen (GL). Rupture in the basement membrane (BM) lining and visceral muscle (VM) putatively led to escape of TcaB to the body cavity (BC) or hemocoel.

bacterial toxins such as α-Xenorhabdolysins (Xax) pore forming toxin complex of *Xenorhabdus nematophila*, BteA cytotoxin (exported via type III secretion system) from *Bordetella* spp., LegC3 effector from *Legionella pneumophila* and cysteine proteinase staphopain B (SspB) from *Staphylococcus* spp. (Ahuja *et al*., 2021).

c. Putative gut receptors for orally active toxins:

After extensive bioassay and histopathology analyses, we identified two gut-active toxins, i.e. Txp40 and TcaB from the Indian strains of *Photorhabdus* spp. Using an *in silico* protein-protein interactor study, it was predicted that Txp40 may dock with different gut receptor proteins from *H. armigera* such as cadherin (CAD), ATP-binding cassette transporters subfamily C (ABCC), aminopeptidase N1 (APN1) and alkaline phosphatase (ALP), that are putatively located in the cell membrane of midgut epithelial cells of *H. armigera*. The electrostatic bonds (interaction energy up to 575 $kJmol^{-1}$) between Txp40-CAD, Txp40-ABCC2, Txp40-APN1 and Txp40-ALP complexes were 6, 4, 1 and 9 in number, respectively; the hydrogen bonds (interaction energy up to 150 $kJmol^{-1}$) between identical ligand-receptor complexes were 15, 16, 14 and 22, respectively (Shankhu *et al*., 2020). Similarly, TcaB docked with CAD, ABCC2, ALP and APN1 receptors of *G. mellonella* via numerous hydrogen bonds, salt bridges and pi-alkyl

bonds. The docking scores (calculated by total interaction energy in terms of distance-based pair potential per residue, where negative score suggests greater energy) for TcaB-CAD, TcaB-ABCC2, TcaB-APN1 and TcaB-ALP complexes were obtained as –527.54, –220.88, –80.54 and –188.48, respectively, implying the superior thermodynamic stability of the docked models (Santhoshkumar *et al*., 2021; Fig. 5). Further, TcaB also docked (binding energy of ligand-receptor complexes rangedbetween –50.14 to –72.34 kcal mol^{-1}) with candidate gut receptors from *S. frugiperda*, *S. litura* and *H. armigera* (Dutta *et al*., 2021a). Recently, using ligand blot, ELISA and RNAi studies it has been experimentally proved that *P. akhurstii* TcaB binds with membrane proximal domain of CAD protein in the midgut epithelium of *G. mellonella* (Dutta *et al*. 2021b).

Conclusion and future directions:

Due to the existence of diverse agro-ecological zones in the vast country like India, Indian farming system suffers heavily from the incidence of a broad spectrum of insects and pests. Development of insect resistance (in Western corn rootworm *Diabrotica virgifera virgifera*, corn earworm *Helicoverpa zea*, *H. armigera*, *S. frugiperda*, pink bollworm *P. gossypiella* etc.) has become an alarming concern because of the continuous monoculture of transgenic Bt plants (Bt maize, Bt cotton etc.). Therefore, exploring the untapped diversity of *Photorhabdus* toxins originated from the Indian strains may strengthen the pest management repository and will provide an alternative to Bt-dominated pest management tactics. The efficiency of the EPN or its symbiont bacterium as biocontrol agent rely on an optimum and narrow spectrum of moisture and temperature in soil. Additionally, EPN's application for foliar pest management has been limited because of IJs' sensitivity to ultraviolet radiation, desiccation and extreme temperatures. In view of this, efforts were undertaken to replicate the insecticidal activity of *Photorhabdus* toxins in heterologous microorganism such as *E. coli*. To date, a number of toxin candidates were characterized from *P. luminescens* W14 and TT01 (ffrench-Constant *et al*. 2007; ffrench-Constant and Dowling 2014; Sheets and Aktories 2017). Nevertheless, the sequences, conserved signatures and mechanism of action of the toxin candidates we characterized from *P. hindustanensis* and *P. akhurstii bharatensis* differed greatly when compared with the identical candidates from *P. luminescens* W14/TT01. Notably, bacterial genomes exhibit a greater degree of genetic/molecular variation compared to other organisms and astonishingly, strains corresponding to the identical species may confer a considerable 3 to 4% difference in their genomes (Ochman *et al*., 2000; Konstantinidis *et al*., 2005).

Our investigations unravel the structure and function of orally active *Photorhabdus* toxins in different Indian strains of *Photorhabdus* spp. By carrying out the immunological and toxicological investigations, we propose that *P. akhurstii* TcaB and Txp40 can negatively alter host insects' innate defense responses and function as a cytotoxin. Additional experiments are required to the establish the probable immunomodulatory property of TcaB and Txp40. *P. akhurstii* TcaB

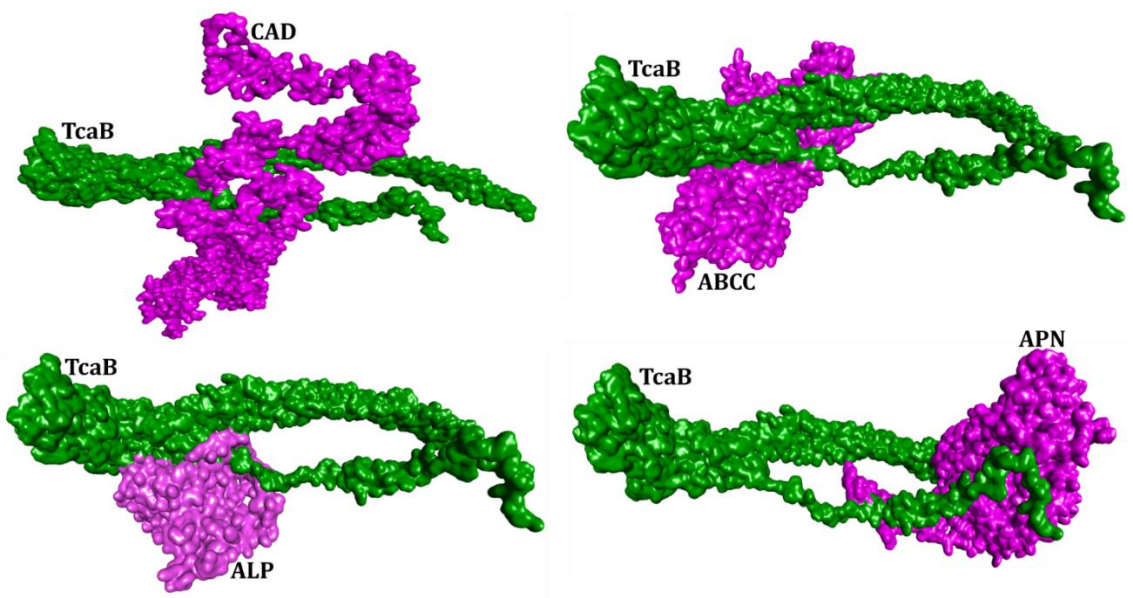

Fig. 5. Simulated binding site interactions of *P. akhurstii* TcaB and *G. mellonella* midgut proteins – CAD, ABCC2, ALP and APN1. Numerous hydrogen bonds, salt bridges and pi-alkyl interactions were detected in TcaB-receptor interface. Protein-protein interactor analysis was carried out in PatchDock webserver (https://bioinfo3d.cs.tau.ac.il/PatchDock/) and docked models were visualized via Discovery studio (v. 4.2). Best models were screened out according to the geometric shape complementary score and minimum energy potential between interacting molecules.

homologues were detected in a broad range of bacterial genera suggesting that Tc genes are indispensable in bacterial lifestyle and their evolutionary processes. TcaB and Txp40 were predicted to be exported from bacterial cell by the bacterial type III secretion system suggesting their effector-like nature. Owing to their smaller molecular mass (63 and 37 kDa), TcaB and Txp40 are suitable candidates for incorporating in molecular breeding of crops for insect resistance. It is worth mentioning that a plant-optimized version of a smaller subunit of *P. luminescens* Tc holotoxin, i.e. TcdA (transcription of the target was enhanced by adding 5′ and 3′ untranslated region (UTR) flanking sequences of *Nicotiana benthamiana* osmotin gene) was transgenically expressed in *Arabidopsis thaliana* that conferred substantial resistance to the larvae of *M. sexta* and *D. undecimpunctata* (Liu *et al*., 2003). There exist many unresolved queries related to the binding of toxin protein to target host cells, their delivery into, and the action of the biologically active components in the host cells. Any successful use of *Photorhabdus* gut-active toxins in agriculture warrants some basic research on the target receptors in the insect midgut. In view of this, the following approaches are advised to identify the potential gut receptors of TcaB/Txp40 protein in different test insects:

- A detailed bioinformatics analyses of CAD, APN, ALP and ABCC across the insect genera to predict toxin binding domains,
- Protein expression of truncated receptors and real-time receptor-ligand binding analysis by western blotting and ELISA,
- RNA/DNA isolation, cloning and characterization of the receptors from different test insects,

- CRISPR-Cas9/RNAi-based knockout/knockdown of selective toxin binding domains in test insects and behavioral/biochemical assays of mutant insects upon oral administration of TcaB/Txp40 toxin,
- RT-qPCR-based comparative expression profiling of receptors in control and mutant insects.

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