Review

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Bacillus thuringiensis subsp. *israelensis* at the Public Health–Ecology-Biotechnology Nexus: From Larvicidal Precision to Protein Delivery Platform Potentials

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Review

Bacillus thuringiensis subsp. *israelensis* at the Public Health–Ecology-Biotechnology Nexus: From Larvicidal Precision to Protein Delivery Platform Potentials

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Abstract

Bacillus thuringiensis subsp. *israelensis* (Bti) is the most widely used biological larvicide for mosquito control worldwide and a cornerstone of environmentally sustainable vector-management programs. Its long-term global deployment reflects a well-characterized balance between public-health benefit and manageable ecological tradeoffs within integrated vector management (IVM) frameworks. Bti combines high larvicidal efficacy, operational simplicity, and strong target specificity, resulting in an exceptional safety profile for humans and vertebrate wildlife. Decades of laboratory and field studies demonstrate that Bti is biologically selective rather than ecologically inert, with reproducible yet context-dependent effects confined to closely related non-target aquatic dipterans. This review links the molecular and toxicological foundations of Bti to its operational performance, ecological selectivity, resistance-mitigating properties, and sustained utility in mosquito-control programs. Beyond its established larvicidal function, Bti's prokaryotic insect larvicidal organelle (PILO) represents an underexplored platform for heterologous intracellular protein assembly. Its dense packing, structural stability, and resistance to environmental and biochemical stress indicate an evolutionary specialization for high-capacity protein storage during sporulation. These properties support noncanonical applications in biomolecule storage and stabilization and motivate cautious exploration of environmentally responsive protein release strategies. Although significant mechanistic and translational challenges remain, particularly with respect to cargo trafficking, modularity, and purification, the architectural principles that have enabled effective mosquito control provide a strong foundation for extending PILO-based platforms beyond larvicidal applications.

Keywords: *Bacillus thuringiensis* subsp. *israelensis*; Bti; mosquito control; integrated vector management; IVM; non-target effects; Cyt1Aa1; prokaryotic insect larvicidal organelle; PILO

1. Introduction

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Vector-borne diseases caused by viral and parasitic pathogens remain a major global public health threat, placing over half of the world's population (~4,000,000,000) at risk and accounting for a substantial fraction of the global infectious disease burden [1–3]. These pathogens are transmitted by a diverse array of arthropod vectors, including mosquitoes, blackflies, tsetse flies, fleas, ticks, lice, sandflies, and triatomine bugs, and they disproportionately affect tropical and subtropical regions where favorable ecological conditions intersect with socioeconomic vulnerability and limited public health infrastructure [1–3].

Among these vectors, mosquitoes comprise the most epidemiologically significant group because of their near-global distribution and capacity to transmit an exceptionally broad range of viruses, protozoans, and helminth worms [1,2]. Climate-driven changes in temperature, precipitation, and habitat suitability can enhance mosquito survival, accelerate pathogen development, and prolong transmission seasons, facilitating both latitudinal and altitudinal range expansion [4–8]. At the same time, rapid urbanization, human mobility, and the spread of invasive vectors such as *Aedes aegypti*, *A. albopicticus*, and *Anopheles stephensi* continue to destabilize historically predictable disease transmission patterns [3,8,9].

The public health burden imposed by mosquito-borne disease is further compounded by the convergence of multiple vector-borne infections within the same geographic regions. A notable example is onchocerciasis, caused by *Onchocerca volvulus* and transmitted by blackflies (*Simulium* species), which remains largely concentrated in Africa and Yemen despite substantial elimination successes [10–14]. At the same time, broadly effective vaccines remain difficult to develop and are unavailable for many mosquito-transmitted agents of disease, underscoring the continued importance of durable, environmentally sustainable vector-control strategies [15–17].

Within this broader framework, the eco-friendly bacterial mosquito larvicide, *Bacillus thuringiensis* subsp. *israelensis* (Bti), has retained its status as the most widely deployed and ecologically defensible microbial larvicide for mosquito control worldwide [18]. It is noteworthy that Bti also played a historically important role in large-scale *Simulium* larval control during early onchocerciasis programs and continues to be used selectively for blackfly suppression in specific settings [19–21]. Following its isolation in the late 1970s, Bti underwent extensive laboratory and field toxicological evaluation, which demonstrated negligible risk to humans, vertebrate wildlife, and most non-target organisms. These findings led to regulatory approval by agencies such as the U.S. Environmental Protection Agency (EPA) and endorsement by the World Health Organization (WHO) [22–25].

This review provides a general overview of the applied use of Bti in mosquito control, linking its molecular and toxicological underpinnings to real-world larvicidal performance in operational settings. Emphasis is placed on how Bti's toxin composition and modes of action translate into reliable field efficacy, high target specificity, a low propensity for resistance development, and compatibility with integrated vector management (IVM) programs. Finally, we consider the potential relevance of the PILO architecture to noncanonical commercial applications, for example, cold chain–independent platforms for mucosal vaccine delivery.

2. General Biology of Bti

Bti is a Gram-positive, aerobic, spore-forming bacterium belonging to the *Bacillus cereus sensu lato* group [26]. Like other *B. thuringiensis* (Bt) subspecies, Bti undergoes a biphasic life cycle consisting of vegetative growth followed by sporulation under nutrient-limiting conditions. During sporulation, the bacterium produces a parasporal body, recently characterized as a prokaryotic insecticidal larvicidal organelle [PILO], composed of crystalline protoxins, most notably Cry4Aa1, Cry4Ba1, Cry11Aa1, and Cyt1Aa1, that are packaged separately from the endospore and remain biologically inert until ingested by susceptible mosquito larvae [27]. These larvicidal proteins are encoded on the large plasmid pBtoxis, and the composite toxin profile distinguishes Bti from other Bt subspecies by conferring potent, dipteran-specific larvicidal activity [28].

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2.1. General Structural Features of Bti's Cry and Cyt Toxins

The structural and biochemical features of Bti's principal Cry and Cyt toxins have been studied extensively, and discussed in considerable detail eslewhere [29–35]. Briefly, most Cry toxins adopt a conserved three-domain fold in their activated state. Domain I is an alpha-helical bundle that undergoes major conformational rearrangement during membrane insertion and pore formation; domain II is beta-sheet rich and contains the variable surface loops that contribute to receptor recognition and target specificity; and domain III contributes to receptor interaction, oligomerization, and structural stability [29,32,33]. Structural studies of full-length toxins and naturally occurring nanocrystals show that crystal packing is an encoded property of the protoxin architecture itself, rather than an incidental result of crystallization [30,32].

The Cyt family of proteins are smaller and structurally distinct from the three-domain Cry family, instead belonging to beta-pore-forming toxin folds [29,30,35]. Crystal structures of activated Cyt toxins reveal compact alpha/beta architectures with exposed hydrophobic regions that promote direct lipid binding. Nonetheless, Cry and Cyt toxins represent complementary molecular solutions to membrane perforation: the former being highly receptor-driven and specific, whereas the latter being more lipid-oriented and broadly membrane interactive [29,30,35].

2.2. Mosquito-Larvicidal Activity of Bti's Cry and Cyt Proteins

Upon ingestion of the PILO by mosquito larvae, Bti crystals are solubilized and proteolytically activated in the highly alkaline larval midgut of mosquitoes. Proteolytically activated Cry toxins then bind to receptors on the brush-border membrane of epithelial microvilli, including cadherins, aminopeptidase N, alkaline phosphatases, and alpha-amylase, before oligomerizing and inserting into the membrane to form pores that drive osmotic imbalance, epithelial disruption, and larval death [34–37].

Cyt1Aa1 differs fundamentally from the Cry toxins in its mode of action. Rather than depending on a defined protein receptor, activated Cyt1Aa behaves as a highly lipophilic toxin that associates with membrane lipids [29,30,35,37]. Interestingly, to avoid premature lytic activity during synthesis in Bti and in heterologous bacterial hosts, the accessory chaperone protein P20 coded by the *cry11A1a* operon in pBtoxis [28] is required to promote proper Cyt1Aa1 crystallization and prevent membrane damage in the producing bacterium [28,36]. Although Cyt1Aa1 is markedly less toxic to mosquito larvae when evaluated alone, its biological importance lies in its strong synergistic activity with Cry4Aa1, Cry4Ba1, and Cry11Aa1 against mosquito and blackfly larvae, as well as its ability to suppress or delay the evolution of resistance in exposed populations [35,37]. In this regard, Cyt1Aa1 is a remarkable and indispensable component of Bti's composite PILO [27].

It is important to note that the synergistic capacity of Cyt1Aa1 extends beyond the native Bti Cry toxins. Cyt1Aa1 can restore or enhance the activity of the *Lysinibacillus sphaericus* binary toxin, Tpp1Aa1/Tpp2Aa1 (formerly BinA/BinB), against intrinsically refractory or resistant mosquito populations, including *Aedes aegypti* and resistant *Culex quinquefasciatus* [38–42]. Earlier models proposed that Cyt1Aa1 acted primarily through detergent-like membrane disruption or the formation of small, nonspecific pores. However, more recent structural and mechanistic studies support a more complex process involving membrane-bound assemblies and context-dependent insertion behavior [30,43–47]. In addition to its intrinsic membrane activity, Cyt1Aa1 can physically associate with heterologous toxins, functioning as a surrogate membrane anchor that facilitates the entry and activity of Cry proteins in resistant larvae lacking canonical Cry-associated receptors [37]. This cooperative, receptor-independent mechanism not only enhances larvicidal efficacy but also contributes to the resistance-mitigating properties that distinguish Bti formulations.

3. Ecological Selectivity, Persistence, and Integrated Use of Bti

Bti remains one of the most selective and environmentally compatible microbial larvicides available for mosquito and blackfly control [18]. Decades of laboratory, mesocosm, field, and post-

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registration studies demonstrate that it combines strong larvicidal efficacy with a constrained non-target impact profile and exceptional vertebrate safety [22–25,48,49]. Importantly, Bti is biologically selective rather than ecologically inert, i.e., measurable non-target effects are largely restricted to a narrow subset of aquatic dipterans and are typically localized, environmentally contingent, and reversible under appropriate management [24,25,50,51].

Within IVM frameworks, these ecological attributes translate into clearly defined operational parameters governing when and how Bti is deployed. Central among these is strict target life-stage specificity, as Bti intoxication occurs only in actively feeding larval stages of mosquitoes and black flies. Effective use, therefore, requires surveillance-based confirmation of larval presence, density, and developmental stage prior to application, inherently favoring threshold-driven interventions over calendar-based spraying and reinforcing adaptive decision-making principles central to IVM [22,25].

Habitat-specific constraints further shape Bti deployment. Aquatic systems vary widely in flow regime, organic content, depth, and hydrological connectivity, all of which influence toxin dispersion, persistence, and larval exposure. In lotic systems, including fast-flowing rivers that support *Simulium* breeding, operational success depends on formulation choice, precise application timing, and repeat dosing to compensate for dilution and downstream transport [22,25]. These constraints were well recognized in historical onchocerciasis control programs, where Bti was deployed as a larval control tool adapted to local hydrology rather than applied uniformly across landscapes [10,11,19–21].

Application rate and timing, therefore, represent additional IPM-relevant parameters. Bti doses are adjusted according to larval density, species susceptibility, and habitat characteristics rather than applied as fixed concentrations. Because Bti exhibits limited residual activity, effective control relies on precise temporal targeting and post-treatment monitoring rather than prolonged environmental persistence; an attribute that simultaneously enhances ecological safety while necessitating adaptive management [22,25].

3.1. Nontarget Sensitivity Boundaries: Aquatic Invertebrates and the Chironomid Effect

At operationally relevant concentrations, laboratory bioassays and field studies consistently demonstrate that cladocerans, copepods, mayflies, caddisflies, aquatic beetles, and most other non-dipteran invertebrates exhibit little or no measurable sensitivity to Bti [24,51]. The principal non-target exception comprises closely related nematoceran dipterans, particularly chironomid midges, whose susceptibility reflects shared physiological traits with mosquitoes, including alkaline midgut conditions and compatible membrane physicochemical properties [24,50–52].

Within chironomids, sensitivity varies by species and exposure regime. Repeated or intensive applications, especially in small or weakly connected wetlands, can cause short-term reductions in larval abundance [50–53]. These effects are generally transient, with recovery occurring within weeks via recolonization and rapid generation turnover [51,53,54], and impacts remain largely confined to closely related dipteran taxa rather than propagating across broader aquatic invertebrate communities [24,51,54]. Sublethal and life-history effects, including delayed development, reduced emergence success, smaller adult size, and decreased reproductive output, have been most consistently documented under frequent or multigenerational exposure [24,54]. Importantly, these responses are habitat-dependent and reversible, rather than indicative of persistent ecological disruption [24,51,54–60].

The ecological relevance of chironomid sensitivity extends beyond individual taxa, as these insects contribute to organic-matter processing and recycling, sediment mixing, nutrient redistribution, and aquatic–terrestrial energy transfer, while also serving as prey for fish and insectivorous birds [59,60]. Consequently, localized suppression in frequently treated, poorly connected systems may generate indirect trophic effects, whereas larger or hydrologically connected wetlands typically exhibit faster recolonization and weaker ecosystem-level responses [51,54,62]. Collectively, the literature converges on a consistent conclusion: chironomids represent a predictable and biologically meaningful non-target of Bti, but associated population-, life-history-, and

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ecosystem-level effects are generally localized, manageable, and reversible when use is surveillance-guided [24,51,59,60].

3.2. Human and Vertebrate Safety: Mechanistic and Empirical Evidence

Toxicological, ecological, and regulatory evaluations demonstrate that Bti is non-toxic and non-pathogenic to humans and vertebrate wildlife, including fish, amphibians, birds, reptiles, and mammals [22–25]. The mechanistic basis for this safety lies in fundamental physiological incompatibilities: Bti crystalline protoxins require highly alkaline midgut conditions for solubilization and proteolytic activation, whereas vertebrate digestive systems are acidic and rapidly degrade any solubilized proteins. In addition, vertebrate intestinal epithelia lack the receptor environments and membrane physicochemical properties required for Cry- and Cyt-mediated pore formation [22,34,37].

Consistent with this framework, mammalian toxicity studies have failed to establish LD_{50} values even at maximum testable doses, and long-term feeding assays reveal no evidence of carcinogenic, reproductive, developmental, or systemic toxicity [23,48]. These findings underpin risk assessments by the U.S. EPA, the WHO, and other regulatory authorities, leading to Bti's classification as a reduced-risk larvicide suitable for public-health use [22,23,49]. Large-scale epidemiological studies and health-surveillance programs following operational applications in North America and Europe report no significant acute or chronic adverse health outcomes in exposed populations [63,64], with occasional minor symptoms attributed to formulation components or particulate exposure rather than intrinsic Cry or Cyt toxicity [63,64].

Laboratory studies reporting cytotoxic effects in mammalian or human cell lines involve highly artificial exposure scenarios, typically requiring direct contact with concentrated spores or products of vegetative outgrowth resembling factors produced by members of the *Bacillus cereus sensu lato* group rather than insecticidal Cry or Cyt proteins [65]. Exposure levels in these assays far exceed realistic environmental or occupational concentrations, and comparable effects have not been demonstrated in vivo or detected through epidemiological monitoring [63,65]. Environmental fate data further support vertebrate safety: Bti toxins do not bioaccumulate or biomagnify and generally degrade under field conditions [22]; fish and amphibian LC_{50} or adverse-effect thresholds exceed operational doses [62]; and long-term monitoring has not detected population-level vertebrate declines in treated wetlands. Emerging behavioral endpoints, such as altered exploratory behavior in zebrafish at environmentally relevant concentrations, do not contradict established safety conclusions but suggest complementary tools for refining ecological risk assessment under intensive exposure scenarios [67]. Taken together, mechanistic, toxicological, epidemiological, and ecological evidence converges on the conclusion that Bti poses negligible risk to human health and vertebrate wildlife when used according to established guidelines [68].

3.3. Evaluating Concerns of Broadened Toxicity Due to Lipophilic Cyt1Aa1

Concerns that the lipophilic Cyt1Aa1 component of the parasporal inclusion body could broaden host range or compromise ecological safety are not supported by available evidence [30,35,45–47]. Cyt1Aa1 remains functionally contained within the mosquito and blackfly intoxication pathway through three linked constraints: alkaline midgut-dependent activation, dependence on specific membrane physicochemical properties absent in most non-dipteran taxa, and its role as a membrane-associated surrogate receptor that enhances Cry binding without expanding taxonomic activity [22–24,35,37,69–76]. Apparent indications of broadened toxicity largely arise from artificial membrane systems or supraphysiological exposure scenarios that bypass realistic Cyt1Aa1 exposure routes [43–47]. Under operational conditions, Bti has not been shown to exert meaningful Cry or Cyt toxicity toward lepidopterans, coleopterans, crustaceans, mollusks, or arachnids [24,46].

3.4. Governance, Long-Term Ecological Tradeoffs, and Sustainable Deployment

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The balance between ecological selectivity and public-health benefit shapes how Bti is deployed across regulatory settings. In highly regulated temperate wetland systems, including California and Western Europe, Bti use emphasizes surveillance-guided applications, spatial precision, and adaptive management in response to observed non-target effects [18,23,25,51,68]. In contrast, in tropical and subtropical regions with persistent mosquito-borne disease burdens, Bti is incorporated as a core disease-prevention tool due to its effectiveness, low logistical requirements, and reduced reliance on broad-spectrum chemical insecticides [22–25,77–79]. Across regions, differences in governance reflect how ecological risk is weighed relative to disease burden rather than disagreement over Bti's selectivity or safety [18,51,80,81].

Environmental persistence adds a temporal dimension to these considerations. Field studies demonstrate that Bti spores and toxins can persist in sediments and decomposing plant material for extended periods, resulting in low-level chronic exposure beyond active treatment windows [82]. Ecosystem-level studies indicate that repeated larvicidal interventions can reduce aquatic insect emergence and associated energy transfer to insectivorous vertebrates, particularly in small or weakly connected wetlands [51,83,84]. Comparative experience with *Bacillus thuringiensis* subsp. *kurstaki* illustrates how cumulative exposure, even to a biologically selective microbial insecticide, can generate indirect ecological and evolutionary effects not readily detected under short-term or small-scale application regimes, including delayed reductions in non-target lepidopteran populations and altered food availability for insectivorous birds [83,84]. These findings underscore the importance of long-term, surveillance-guided deployment of Bti within IVM frameworks.

Although Bti's multitoxin Cry/Cyt architecture confers strong resistance-mitigating properties, laboratory selection and population-genomic studies demonstrate that sustained exposure can still drive reduced susceptibility in mosquitoes, underscoring the need for resistance-aware deployment within IVM programs [35,37,39–42,70]. IVM therefore remains central to Bti's continued utility, incorporating surveillance-guided use, habitat-specific targeting, and coordination with complementary control strategies to maximize public-health benefits while maintaining predictable and manageable ecological tradeoffs [85–87].

Taken together, Bti functions within IVM as a larvicide whose effectiveness is governed by larval biology, habitat characteristics, precise application timing and dosing, surveillance-guided decision-making, and resistance-aware deployment. These parameters ensure that Bti maximizes public-health benefit while maintaining predictable and manageable ecological tradeoffs, consistent with WHO-endorsed IVM principles [10,11,22,25].

4. Translational Robustness of Bti: From Commercial Larvicides to Programmable Protein Platforms

The sustained global deployment of Bti as a mosquito larvicide reflects the structural robustness, specificity, and reliability of its native PILO (**Figure 1**), which accounts for ~25% of cellular dry mass [18,27,88,90] In addition to the PILO, Bti is capable of synthesizing supplementary cytoplasmic inclusions of comparable size, allowing functional augmentation without perturbation of the native toxin architecture. For example, a recombinant Bti strain producing an inclusion of *L. sphaericus* binary toxin Tpp1Aa1/Tpp2Aa1 (~1 μm) within a sporulated parental background that retains the native PILO, also ~1 μm, demonstrates the bacterium's capacity to spatially accommodate three large intracellular structures (PILO, Tpp1Aa1/Tpp2Aa1, and spore) within the cytoplasm prior to autolysis [90]. This recombinant strain represents the most potent bacterial mosquito larvicide described to date, exhibiting a 21- to 32-fold increase in activity against medically important mosquito species relative to parental Bti and *L. sphaericus* strains [90]. Comparable enhancements achieved through the incorporation of Cry11B, or to a lesser extent, a heterologous chitinase [91], further underscore how Cyt1Aa-mediated synergism can be strategically exploited within the conserved molecular design of the PILO.

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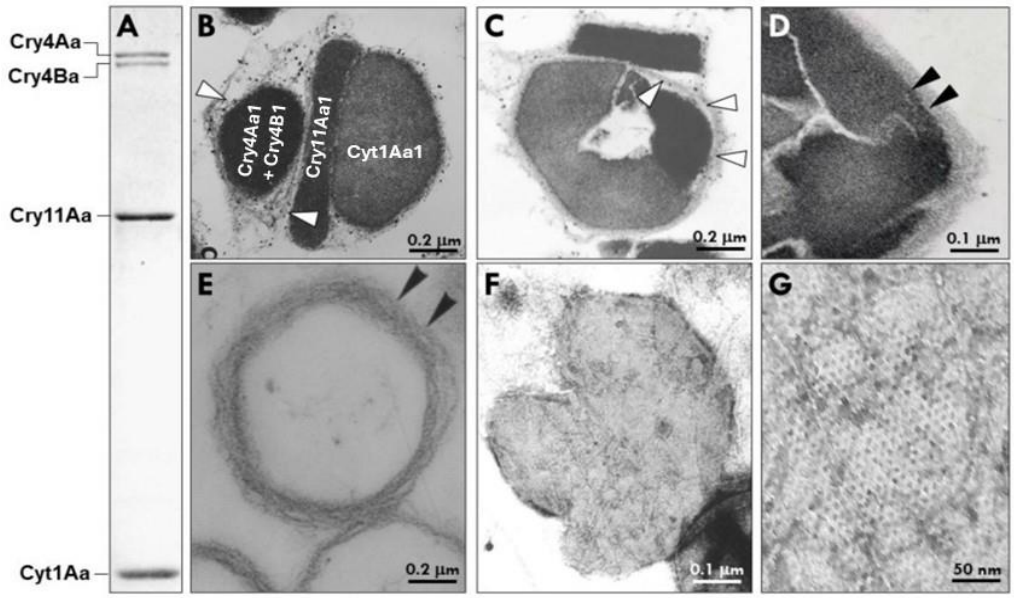

Figure 1. Protein profile and structural features of Bti's PILO. (**A**) SDS-PAGE showing the major crystal proteins of the PILO, i.e., Cry4Aa1 (135 kDa), Cry4Ba1 (128 kDa), Cry11Aa1 (65 kDa), and Cyt1Aa1 (27 kDa). (**B,C**) Representative transmission electron micrographs of Bti parasporal bodies, with arrows indicating the multilaminar fibrous matrix (MFM) surrounding individual crystal proteins (white arrowheads indicate the MFM inner layer). (**D**) Black arrowheads indicate the tightly packed MFM surrounding the PILO at the end of sporulation. (**E**) Purified MFM after dissolution of insecticidal proteins in alkaline buffer. (**F,G**) Negatively stained MFM following protein dissolution, revealing a hexagonal arrangement of the pores. Reproduced from Rudd et al. *Biology* 2023, *12*, 1421, CC-BY 4.0.

Multiple *Bacillus* species are now well established as probiotics. Among these, *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus clausii*, and *Bacillus licheniformis* are the most widely studied and commercially utilized, with demonstrated benefits for gut health, immune modulation, and pathogen suppression [55–57]. Additional species such as *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus pumilus* have also been investigated for probiotic-associated functions, including antimicrobial metabolite production [57]. Although the probiotic capacity of *B. thuringiensis* strains has not been extensively investigated, emerging evidence suggests a non-canonical role for Bti in animal nutrition and gut health modulation [58]. Recent experimental work in broiler chickens has demonstrated that dietary supplementation with Bti can significantly reduce intestinal crypt depth and increase the villus-to-crypt ratio, indicating improved intestinal integrity and epithelial efficiency, without altering systemic immune markers or reproductive development [58]. These findings suggest that Bti may exert localized effects on enterocyte turnover and gut homeostasis rather than acting as a classical immune-stimulating probiotic, thereby expanding its functional profile into the domain of gut-centric microbial modulators [58].

Beyond its established larvicidal role and potential probiotic-associated functions, the Bti's PILO (**Figure 1**) represents a largely underexplored biological structure with relevance beyond insect control. Structurally, the PILO is a self-assembling, microbial factory characterized by extreme packing density and exceptional resistance to environmental, physical, and biochemical stresses. The porous multilamellar fibrous matrix, whose ultrastructure remains to be fully defined, exhibits pronounced stability under both acidic and alkaline conditions and is resistant to enzymatic degradation, including proteolysis [27,92]. These attributes reposition the PILO not merely as a toxin delivery structure, but as a robust and potentially programmable protein scaffold with broader biotechnological relevance [27].

Genetic uncoupling of parasporal inclusion formation from larvicidal activity enables reinterpretation of the PILO (~1 mm) as a neutral intracellular protein reservoir. The ability of Bti to accumulate extraordinarily high protein loads during sporulation without compromising viability is

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unusual among bacteria and conceptually parallels engineered protein compartments such as encapsulins (~24-42 nm) and bacterial microcompartments [93–100], albeit at a substantially larger scale. At approximately 24–45-fold greater size than nanoscale protein cages, the PILO provides enhanced payload capacity and prolonged intracellular persistence, features that are challenging to replicate using synthetic scaffolds.

These properties support expanded applications centered on biomolecule storage and stabilization. Sporulation-derived proteinaceous inclusions are intrinsically adapted to preserve molecular integrity through dormancy, desiccation, and environmental stress, offering a biological alternative to lyophilization or synthetic encapsulation for stabilizing labile enzymes, antigens, or industrial proteins, including heavy metal-binding proteins [101–105]. Such approaches are particularly attractive in resource-limited settings where cold-chain–independent preservation remains a significant logistical barrier. Beyond passive stabilization, the physicochemical resilience of the PILO matrix supports cautious consideration of its potential as a delivery scaffold for mucosal antigens, especially in the context of enteric pathogens, where antigen instability, proteolytic degradation, and inefficient epithelial uptake remain limiting factors [102–105].

Translation of PILO-based systems into non-larvicidal applications will depend on advances in several key areas: (i) deletion of *cry* and *cyt* genes in pBtoxis; (ii) elucidation of the mechanisms governing incorporation and trafficking of heterologous proteins within the multilamellar matrix; (iii) identification of PILO-associated components that could be repurposed as modular cargo-packaging elements; and (iv) evaluation of practical considerations such as scalability and protein recovery. Indeed, in our current work, we are beginning to uncover factors required for trafficking of proteins into the PILO and identifying roles of proteins other than Cry and Cyt that associate with the multilamellar matrix. These proteins include pBtoxis-coded Bt152, which is required for stability of the PILO, and Bt075, which, intriguingly, shares sequence and structural homology to encapsulin shell proteins [27]. As an example, we have expressed a *Bt075-green fluorescent protein (gfp)* gene chimera in a Bti strain lacking the PILO, and have observed discrete intracellular and purified particles that are below the limit of resolution of the light microscope (<0.2 mm), suggesting the Bt075 indeed forms an encapsulin shell (unpublished data). In this regard, the evolutionary complexity of Bti's PILO is underscored by the apparent repurposing of an encapsulin-like shell protein for structural protection at a substantially larger organizational scale, highlighting novel mechanisms that may be exploited in the design of modular, non-larvicidal PILO-based systems [27].

Finally, insofar as the translational utility of the PILO can be realized, several practical considerations appear readily tractable. Bti completes sporulation and subsequent autolysis within approximately 48–96 h, efficiently releasing spores together with parasporal inclusions and any associated recombinant PILO assemblies, a production cycle that is both cost-efficient and compares favorably with many heterologous protein expression platforms. Established particulate fractionation approaches developed in Bti research, including density-based centrifugation methods, already provide robust means for separating parasporal inclusions from spores, cellular debris, and soluble proteins [90,92,106]. Importantly, the exceptional protein packing density of the PILO suggests capacity not only for high-level accumulation of individual cargos, but also for mosaic packaging of multiple distinct proteins within a single assembly, enabling multifunctional or combinatorial applications. Although the feasibility of PILO-derived platforms beyond larvicidal applications remains to be demonstrated, several features support their consideration as a distinctive and currently underutilized class of microbial protein scaffolds. These include the relative ease of conventional molecular engineering and strain development, the spontaneous release of recombinant PILO assemblies upon autolysis, and the availability of established downstream processing approaches.

5. Conclusions

The sustained global success of Bti as a mosquito larvicide reflects not only the biological efficacy of its toxin repertoire but also the inherent robustness, scalability, and reliability of its PILO. When

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considered independently of larvicidal function, the PILO emerges as an underappreciated intracellular protein assembly factory whose evolutionary optimization for high-density protein accumulation, stability, and environmental persistence provides a compelling foundation for broader translational applications. Advances in synthetic biology, protein engineering, and systems microbiology now enable this structure to be reconsidered as a programmable protein scaffold for biomolecule storage, stabilization, and environmentally responsive release, and, more speculatively, for mucosal antigen delivery. Importantly, the architectural principles that have enabled decades of safe, effective, and large-scale vector-control deployment may also support translation into non-larvicidal applications without sacrificing environmental compatibility or production feasibility. While significant mechanistic and translational challenges remain, the work summarized here supports the view that the PILO represents not merely a specialized insecticidal organelle, but a versatile microbial protein platform with potential relevance beyond its traditional role in vector mosquito control.

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