Growth variation among *Bacillus thuringiensis* strains can affect screening procedures for supernatant-secreted toxins against insect pests

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**Abstract**

**BACKGROUND:** Supernatant-secreted proteins in *Bacillus thuringiensis* (Berliner) (*Bt*) with insecticidal activity provide an important source of information for discovery of new useful strains and/or entomotoxins. However, physiological variation among isolates might interfere in the detection efficiency of screening procedures on *Bt* collections. The aim of this study was to assess the magnitude of this variation in a sample of isolates from a tropical *Bt* collection, which was gauged through the assessment of their temporal patterns of growth and protein secretion in culture supernatants (SNs), as well as of the corresponding toxicity against fall armyworm (*Spodoptera frugiperda*, JE Smith). Feeding bioassays were performed, with larvae being treated with heated and non-heated total protein extracted from SNs collected at different culture times. Larva mortality and reduction in pupa formation were observed.

**RESULTS:** Intra- and interisolate variations were observed in the temporal patterns of growth, quality and quantity of protein secreted, as well as in insecticidal activity of these SNs, based on larvae mortality and pupation rates. These results suggest that the insecticidal potential of certain isolates can be hidden if comparisons are done on the basis of the same number of cells in the culture and/or the same culturing time.

**CONCLUSIONS:** Methods of screening *Bt* collections on the basis of feeding bioassays can be misleading with regards to identifying more promising isolates for biocontrol purposes if physiological differences are not considered. The consequences and implications of these findings for the development of experimental systems that depend on toxicity bioassays to identify alternative *Bt* strains and entomotoxins with practical applicability have been discussed.

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**Keywords:** biological control; entomotoxins; feeding bioassays; first-tier screening; culture supernatant; *Spodoptera frugiperda*; secreted proteins; physiological variation; culture collections

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1 **INTRODUCTION**

The biological control of insect pests appears to be a practical alternative to minimise the economical, environmental and public health impact of chemical pesticide applications.1 For the past six decades, *Bacillus thuringiensis* Berliner (*Bt*) has been the main source of an array of entomotoxins that have provided consistent biological control over several insect and nematode pests of economical importance in agriculture.2 In spite of its affordable costs and environmental safety, this system has not been more widely employed, mostly because of undesired physical and chemical properties of *Bt* sprays that cause lack of toxin stability over the plant and difficult accessibility to the insects in the field.3, 4, 5 Moreover, the development of resistance to *Bt* toxins in populations of crop pests has become a reality, especially owing to the increase in the worldwide use of *Bt*-derived insecticides and plants expressing *Bt* genes, which constitute a continuous source of entomotoxins in a given area.4, 5, 6 Thus, research efforts to uncover new *Bt* strains bearing more effective toxins and/or new specificities remain a constant necessity.1

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The main source of Bt toxins has been parasporal crystalline inclusions which bear the δ-endotoxins known as insecticidal crystal proteins (ICPs). As some insect pests of economic importance are recalcitrant to several Bt δ-endotoxins, entomotoxins secreted into the supernatant of Bt strains, during logarithmic and early stationary phases of growth, were included more recently in the repertoire of alternatives with practical applicability in biological control.8−11 One of these activities, the Vip3A-like toxin, has been recently studied in the ‘crop–pest’ model system of ‘tropical maize–fall armyworm’.12−14

Screening procedures that assess Cry proteins through insect feeding bioassays are simple and efficient, as the δ-endotoxins are accumulated (concentrated) in the parasporal inclusions after a defined culturing time, thereby allowing easy qualitative and quantitative comparisons among isolates15−17 or individual toxins.3,5 On the other hand, the screening of Bt cultures for protein toxins secreted in the culture supernatant (SN), such as Vip6,18 Cry 1f (former Cry V),9 Sip1,11 α-exotoxin (phospholipase C),19 exochitinases20 or enterotoxins,21 appears to be a more difficult task, as it is possible that SNs obtained from different culturing times of a single isolate could generate distinct secreted-protein extracts, with variable entomotoxic activities.14 Therefore, as the best timing for maximal protein secretion (or activity) may not be the same among the isolates under scrutiny, this would cause confounding effects in any comparative procedure aiming at sorting superior Bt isolates and/or toxins. Moreover, different sorts of molecular interaction and post-secretion modification may also occur in the total protein fraction, thereby altering the final insecticidal outcome of the SN toxins. Hence, the objectives of this study were to assess whether there is any significant physiological variation in growth and secretion of proteins into culture medium among Bt isolates, as well as whether the magnitude and characteristics of this variation could interfere with the screening for higher entomotoxin activities in the SN of Bt cultures.

2 MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

The B. thuringiensis (Bt) isolates used belong to a large tropical collection (more than 4700 entries) of the National Research Centre of Maize and Sorghum (CNPMS/EMBRAPA, Brazil), and were selected for this study on the basis of geographical variation. They were obtained from at least nine different locations that span the north-eastern, south-eastern, central-western and southern regions of Brazil (Table 1).22 According to previous work,17,23 their ICP toxicities against fall armyworm larvae were distributed as follows: eight isolates presented mortality levels of 90–100%, two around 75%, one around 30% and four below 10% or null (Table 1). Two foreign Bt strains, HD125 and HD11 (US Department of Agriculture), were also used, both showing 100% larva mortality.

For all experiments, bacterial cells were cultured in a two-step procedure. First, 250 mL flasks containing 50 mL of TB liquid medium14 were inoculated with a loopful of cells from each of the −80 °C glycerol stocks of the Bt isolates and grown at 30 °C and 200 rpm. After 12 h of growth, the amount of cells in culture was assessed by hemacytometer. In the second step, for assessment of growth and total protein secretion in culture, inoculum volumes from the previous 50 mL cultures were adjusted to obtain 10⁶ cells mL⁻¹ per isolate, which were added to 250 mL flasks containing 100 mL of the same medium and grown under the same conditions. At the culturing times indicated in the corresponding figures (see Section 3), 800 μL of each isolate culture was collected under sterile conditions, and the number of cells was assessed by optical density (OD) at 590 nm. These samples were then centrifuged for 5 min at 16,000 × g, and 26.7 μL of each supernatant (SN) was withdrawn to estimate the amount of total protein by the dye binding method,24 using OD at 590 nm and Bradford reagent from Sigma (St Louis, MO), following the manufacturer’s recommendations. A BSA-based standard curve was used to estimate protein concentrations. For both the number of cells and the protein content estimation, proper dilutions and corrections were always performed whenever the spectrophotometer readings fell out of the linearity range of 0.05–0.99. This growth curve experiment was repeated at least once for each isolate, showing very similar results.

2.2 Supernatant processing for feeding assays

For the feeding bioassays with SNs collected at different culturing times, large 2 L cultures in 5 L flasks were set for the HD125, HD11, 474L, 606b and 1109o isolates, which were inoculated from the corresponding precultures at the same initial concentration of 10⁸ cells mL⁻¹ per isolate (final culture volume) and grown under the same conditions as those described above. At each time point (5, 8, 11, 14, 17, 20 and 23 h), 96 mL aliquots were centrifuged at 30,000 × g for 20 min at 4 °C, and its SN was carefully collected to avoid carryover of pelleted cells. The SN had its total protein extracted by ice precipitation, which was performed by the slow stepwise addition of ammonium sulfate to a final concentration of 70%, and left overnight at 4 °C with gentle stirring. The precipitated samples were centrifuged at 30,000 × g for 20 min, with the pellets air dried for 20 min at room temperature and resuspended in 8 mL of PBS buffer (pH 7.5). In order to remove excess ammonium sulphate remaining in the resuspended pellets, the samples were dialysed for 10 h at room temperature against 1 L of the same PBS buffer or water (4 °C-stored solution was used, with one change after 4 h). With this procedure, the total protein fraction

| Table 1. Mortality levels and original locations of the tropical Bt isolates used in the study |
|------------------------|-------------|-----------------|-------------|-----------------|
| Isolate | Crystals | SNs³ | Locality (city) | State | Region |
| 7b1 | 31.1 | – | Limoeiro | Alagoas | NE |
| 344 | 73.9 | 4.8 | Foz do Iguacu | Paraná | S |
| 348 | 83.8 | – | Foz do Iguacu | Paraná | S |
| 426 | 96 | – | Araripe | Ceará | NE |
| 5460 | 100 | – | Cascavel | Paraná | SE |
| 461a | 95.5 | 4.8 | Cascavel | Paraná | SE |
| 474L | 100 | 4.8 | (Unknown) | – | – |
| 520b | 95.6 | 0.0 | Boa Esperança | Minas Gerais | SE |
| 566bpr | 100 | – | (Unknown) | – | – |
| 606b | 0 | 2.4 | (Unknown) | – | – |
| 701 | 100 | – | Assis | São Paulo | SE |
| 702 | 100 | – | Campo Grande | Mato Grosso Sul | CW |
| 739alo | 0 | – | Campo Grande | Mato Grosso Sul | CW |
| 858a | 4.2 | – | Patos de Minas | Minas Gerais | SE |
| 1109o | 77.2 | 4.8 | Rio Verde | Goiás | CW |
| HD125 | 100 | 38.1 | – | USA | – |
| HD11 | 100 | – | – | USA | – |

³ Bioassays using total protein extracted from 12 h supernatants;¹⁴ dashes indicate strains that were not tested for SN toxicity.
from the SNs was concentrated 12 times; quantification was done by the dye binding method. To monitor the thermolabile and thermostable insecticidal activities, two 4 mL aliquots from each SN were differentially treated with temperature: one 4 mL aliquot was immediately placed on ice without heating, while the other was heated at 95 °C for 20 min and set on ice until complete cooling. Both non-heated and heated SN treatments were allowed to equilibrate to room temperature (25 °C) before being applied to the insect diets (see Section 2.3). The difference between the mortality obtained with the non-heated (total activity) and heated aliquots (only thermostable toxins) provided an estimate of the global insecticidal protein activities.

2.3 Insect rearing and toxicity bioassays
Fall armyworm (Spodoptera frugiperda, JE Smith) was the insect pest used as the experimental system. Larvae were collected from the field and reared for several generations on cubes of artificial, semi-solid diets (~1 cm³) at 28–30 °C with a natural photoperiod of 12–14:12–10 h light: dark, depending on the season. The diet composition used in all feeding experiments has been described. For the bioassays, larvae were obtained from ovipositions of female adults obtained regularly from this rearing scheme and subjected to the treatments. SN aliquots of 0.165 mL (enough to wet the entire surface of a diet cube) were used to soak each diet of a set of 24 diet cubes, which were transferred to 100 mL disposable plastic containers (one diet per container). The diets were allowed to air dry for ~45 min before a two-day-old larva was added to each container, which was closed with a semi-transparent plastic lid (a total of 24 larvae per treatment). The larvae were kept at 25–28 °C, and the mortality assessment was performed every 3 days, until the moment at which pupa formation was evaluated. This latter parameter was assessed for all treatments when the control reached ≥50% pupation (~15–17 days on average), by counting the numbers of pupae formed and non-pupa larvae remaining alive. The control treatments were 24-larva sets reared in diet cubes immersed in sterile distilled water. This experiment was performed 3 times.

Statistical chi-square analyses for mortality were performed on a per experiment basis, based on pairwise comparisons (2 × 2 contingency tables) between treatments and control. Each treatment was a combination of isolate, culture time and SN heating. In these experimental conditions, usual levels of mortality for controls ranged from zero to three deaths per set of 24 larvae among experiments (data not shown), so that the number of larvae used as ‘expected’ values for control mortality in the 2 × 2 tables were 2 (dead) and 22 (survived). Given the total number of larvae in each contingency table assessed, a correction for continuity was employed.

2.4 Polycrylamide gel electrophoresis
The total protein composition of temporally collected supernatants (SNs) was analysed by denaturing polycrylamide gel electrophoresis (SDS-PAGE). The gels were loaded on the basis of the same volume per sample, which also reflects quantitative differences among precipitated/resuspended samples. For the HD125 temporal profile of peptides, concentration gradient gel was used; loading buffer was added to 70 µL aliquots of the concentrated proteins, boiled for 3 min, loaded into a 16.0 cm long and 0.75 mm thick vertical gel (BioRad®) and run in 50 mM of Tris-HCl (pH 8.8), 0.192 M of glycine buffer for 16 h at 65 V. The stacking gel was 6.0% acrylamide, 0.125 M of Tris-HCl (pH 6.8), and the resolving gel was a gradient between 7.5% (top) and 18% (bottom) acrylamide, 0.5 M of Tris-HCl (pH 8.3), both with 0.1% SDS. For the other isolates, 12.5% acrylamide gels were used; the loading buffer was added to 20 µL aliquots of the concentrated proteins, boiled and loaded into 7.0 cm long and 1.0 mm thick minigels (BioSystems®), with two isolates per gel. The stacking gel was 4.9% acrylamide, 0.26 M Tris-HCl, and the resolving gel had Tris-HCl at 0.38 M; pH and SDS were the same as above. The gels were run in the same Tris-glycine buffer for 5.5 h at 150 V. The gels were stained with Coomasie Brilliant Blue solution (0.15% CBB-R-250, 50% methanol, 10% acetic acid), and recorded by an ‘Eagle Eye’ photodocumentation system (Stratagene, La Jolla, CA).

3 RESULTS

3.1 Physiological variation in culture among B. thuringiensis (Bt) strains
To assess the extent of a potential variation in physiology among Bt strains, and how it would affect their insecticidal activity, a total of 17 Bt isolates, most from a tropical origin, from various geographical locations in Brazil (Table 1), were studied with regard to their growth curves and temporal profiles of total protein secreted in the corresponding SNs (Fig. 1). For the sake of clarity, only the results for the ten more representative isolates were presented; all others displayed patterns of growth and protein secretion within the range of variation observed in Fig. 1 (not shown). The results indeed showed that a clear-cut physiological variation does exist among isolates grown in liquid culture, in spite of the care taken to ensure the same amount of actively growing cells in culture per isolate to begin with (see Section 2). Considering the time needed to enter into exponential growth, the isolates differed from each other by as much as 7 h (Fig. 1a, horizontal dashed line). Three growth speed groups could be observed: isolates 348, 461a, 474L and 606b started the log phase after 4 h of growth, and HD11 ~1 h after that, constituting the fast growth group; all other isolates comprised the medium growth group, starting log phase at ~7 h, with exception of HD125, which entered exponential growth only after 10–11 h, representing the slow growth group (Fig. 1a). Taking isolates all together, the period with higher variation in terms of number of cells was 12 h of growth, when the OD at 590 nm varied by a factor of about 10 from minimal to maximal values, i.e. from 0.73 for isolate HD125 to 7.29 for isolate 474L (Fig. 1a, vertical dashed line). The strain HD125, which was used in this study as a reference strain for SN toxicity (Table 1), entered into the exponential phase of growth in liquid culture after 12 h and reached the stationary phase at around 22–23 h (Fig. 1a), confirming previous studies.

Such diversity was also apparent on the basis of isolate-specific quantitative profiles of protein secretion into SN (Figs 1b and c). Overall, the isolates showed a trend of an oscillating pattern of protein secretion, with the largest differences occurring at two moments: after 8 h of growth (Fig. 1b) and at around 22 h (Fig. 1c). Amounts of total protein below the control levels were observed in the first ~3 h of growth for all isolates (Fig. 1b), with an increased protein content in the SNs observed only after ~4–5 h. The data also indicated that the levels of protein secreted by each isolate were not strictly associated with the corresponding growth curves. For instance, the isolates 474L and 461A depicted a similar growth pattern, but the amount and profile of protein secreted was very distinct. In general, the patterns of growth and protein secretion were not associated in any way with the corresponding mortality levels of the ICP fraction (Table 1, Fig. 1).
In vitro growth of Bt affects screening for supernatant entomotoxins

Figure 1. Growth and protein secretion profiles of ten Bacillus thuringiensis strains in liquid culture. (a) The number of cells (growth curve) was evaluated through optical density (OD) at 590 nm; the horizontal and vertical dashed lines indicate the timeframe among isolates for the start of exponential phase and the maximal variation in cell density at a single time point of culture respectively. (b), (c) The estimation of the total amount of protein secreted in the SN was assessed by the dye binding method at an OD of 590 nm. The legend for curves and isolates is the same for both graphs. Water was used as the blank for spectrophotometer readings; the value obtained for culture medium served as the baseline (zero) for the other treatments.

Only four isolates (HD125, 344, 461a and 739slo) secreted proteins to the respective SNs at levels above 0.1 mg mL\(^{-1}\) (24 h, Fig. 1c). For the reference isolate (HD125), the amount of secreted protein had a first peak at 16 h, decreased until 18 h and then rose again, until it reached a maximum peak at 22 h (Fig 1c); total secreted proteins at this second peak was about twice as large as at the first peak. In addition, the quantitative variation in the protein content of HD125 SN did not strictly follow the increase in bacterial cell mass, although the maximum peak of secretion roughly coincided with the entry into stationary phase (Fig. 1c). Interestingly, this strain showed the largest amount of protein secreted in its SN, but the lowest number of cells in culture (Fig. 1).

3.2 Temporal variation in total protein secretion in Bt cultures

Qualitative variation in protein secretion was assessed by SDS-PAGE, both within a single strain's culture (HD125), during its exponential growth and early stationary phase, and among different strains (Fig. 2). In the first case it was observed that several proteins of various molecular weights altered their band intensity and relative proportion with time (Fig. 2a). Bands of the expected size for various secreted entomotoxins previously described, such as Vip3A (∼88 kDa), Cry 1I (∼60 kDa), α-exotoxin (or phospholipase-C, ∼34 kDa)\(^{19}\) and exochitinase (∼36 kDa)\(^{20}\) were observed. Moreover, the rich peptide profile observed for HD125 also presented bands of the expected size for other described toxins, Vip1A (∼70 kDa) and 2A (∼45 kDa)\(^{18}\) and Sip 1A (∼38 kDa)\(^{11}\) (Fig. 2a). Qualitative differences in the time course of SN protein content were also observed among isolates, as shown by the electrophoretic profiles of isolates 606b and 1109o (three time points at 3 h intervals) and HD11 and 474L (at 6 h intervals), chosen for comparison and illustration purposes (Fig. 2b). Peptides of the expected size for the Bt toxins mentioned above (Fig. 2a) were observed, although they appeared in different quantities per isolate and culturing time (Fig. 2b, arrows). Although all four of these isolates appeared to be similar for their total protein content estimation (Fig. 1c), they were quantitatively different for various peptides (Fig. 2b), for the mortality levels of their ICPs (Table 1) and for the number of cells in the same culture time, at least until ∼20 h of culture (Fig. 1a).
Figure 2. Gradient SDS-PAGE profiles of the total protein secreted in the culture supernatants of Bt strains. (a) Temporal profile of secreted peptides for HD125 strain from early logarithmic up to early stationary phase. Molecular weights are indicated on the left, with the size of putative secreted entomotoxins (88 kDa Vip3A, 60 kDa Cry 1I, 34 kDa α-exotoxin, 36 kDa exochitinase) indicated by arrows on the right. (b) Profiles of secreted peptides for the four other tropical isolates, compared in a similar time range; protein was extracted at the indicated time points, with 3 h (606B and 1109o) and 6 h (HD11 and 474L) intervals. Arrows on the left indicate the same set of molecular weights as above.

3.3 Temporal variation in supernatant-related insecticidal effects among Bt strains

In order to verify the insecticidal effects of secreted entomotoxins of the five Bt isolates shown in Fig. 2, at different culturing times, fall armyworm feeding bioassays were performed using extracted total protein from supernatants, both under non-heated (SN) and heated (SN-Δ) treatments. As proteotoxins are expected to denature and lose their activity under high temperatures, whereas other non-proteic compounds (e.g. β-exotoxins) retain their toxicity, the objective of heat-treating the SNs was to differentiate thermolabile (proteins) from occasionally coprecipitated thermostable (non-proteic) toxins. As expected by the present working hypothesis, SN and SN-Δ treatments showed significant differences in larvae mortality, depending on the isolate and culture time from which total secreted protein was extracted (Fig. 3). The period of culture in which strain HD125 showed a significantly higher toxicity (chi-square analyses, $P < 0.05$) against fall armyworm (20 and 23 h) coincided with the moment of highest protein secretion in SN, i.e. 22 h (Fig. 1c). On the other hand, HD11 displayed higher levels of mortality only at 23 h culture (Fig. 3), but 7–8 times less protein secreted in the SN (Fig. 1c), in a culture with ~40% more cells than HD125 (Fig. 1a); although at low, non-significant levels, the other isolates showed distinct patterns of oscillating toxicity (Fig. 3), which was not associated with either the respective growth curves or the levels of protein secretion in SN (Fig. 1). Larval mortality of SN-Δ treatments was present and also variable, although its level was always $\leq 16.7\%$ for all isolates/culture times (Fig. 3), which was not statistically different than control, based on pairwise chi-square analyses. The electrophoretic profiles observed for the isolates, showing peptide bands of the expected size for various entomotoxins (Fig. 2), were not compatible with toxicity presented by the SNs. Although the expected-size bands could be seen for all isolates, with some of these bands being visible since 8–11 h (Fig. 2), only HD125 showed an increase in toxicity after 20 h of culture (Fig. 3).

Considering that significant delays in larval development is another possibility of toxic effects to be assessed on Bt SNs, the
percentage reduction in larval pupation in relation to control was also evaluated. The results showed that several non-heated SNs from all tested isolates showed statistically significant reduced pupation, whereas the vast majority of SN-Δ treatments did not. However, in spite of consistency in the control treatments, results from this analysis for the SN treatments were not sufficiently reproducible between replicated experiments. Further work is thus required to sort through the causes of such variation before pupation time can be used as a reliable variable and conclusions can be drawn for this system (see below).

4 DISCUSSION
Integrated insect pest management (IPM) programmes for plant protection must overcome several important challenges, such as (i) ensuring economical viability and efficiency, as well as environmental sustainability of the pest management process, (ii) reducing or eliminating environmental and public health threats caused by the excessive use of chemically synthesised pesticides and (iii) preventing, delaying or overcoming pest resistance development. Decisions about suitable isolates for biocontrol purposes include the use of single or multiple beneficial microorganisms, with different mechanisms of action, and improved formulations and delivery systems as well. Moreover, host specificity is crucial in determining the balance between target pest control and side effects on other non-target species, i.e. whether or not biocontrol strategies will be efficient and environmentally safer. Therefore, alternatives in terms of biocontrol agents (BCAs) and/or their derived active substances are constantly necessary, thereby directing an important part of research towards uncovering strains/isolates bearing more effective toxins and/or new specificities. B. thuringiensis (Bt) strains producing and secreting entomotoxins during logarithmic and early stationary phases are some of these recognised alternatives. By using Bt supernatants on feeding bioassays with tropical fall armyworm (S. frugiperda) larvae as a model system, it is here reported how screening procedures in this context can be significantly affected by temporal variation in growth and physiological aspects of different strains.

Owing to precision and fastness, molecular methods tend to be preferred for the identification and selection of strains, genes and proteotoxins, followed by feeding bioassays as a confirmatory step. Depending on the system under study, a feeding bioassay is also important as a first-tier screening method due to its acceptable efficiency, low costs and wide applicability. In a previous report, the supernatants (SNs) of liquid cultures grown to the same time were employed in feeding bioassays to compare Bt isolates collected from tropical environments of Brazil, with the results showing statistical differences in their larval mortality. However, a concern was raised about whether that culturing time (for that particular inoculum amount) would have been the most appropriate to compare isolates, as the presence of Vip3A-like genes was found to be ubiquitous in tropical Bt germplasm, but the mortality levels for the corresponding SNs were very low (Table 1). The results of the present study clearly suggest that comparisons of SN-related entomotoxic activities on the basis of the same culturing time, or optical density, will certainly be compromised by the physiological variation observed among different isolate cultures (Figs 1 and 2). In other words, as the best timing to collect a SN for its maximal toxicity seems to be a strain-specific characteristic (Fig. 3), comparison among isolates cultured up to the same time may be misleading for many of them in terms of their insecticidal potential. For instance, if isolates had all been cultured up to 20 h, the insecticide potential of HD11 would not have been considered; if they had been cultured up to 17 h, both HD125 and HD11 would have been lost (Fig. 3). Taken together, these results would be in agreement with data showing that expression, secretion and activity of major SN-secreted toxins of Bt are identifiable in a time

![Figure 3. Mortality of fall armyworm larvae in toxicity bioassay using artificial diets soaked in total protein extracted from supernatants. Non-heated and heated proteins are indicated by 'SN' and 'SN-Δ' respectively. Sets of 24 larvae (replicas) were used per treatment, per experiment; error bars correspond to three replications of the whole experiment. Statistics was performed on a per experiment basis by pairwise chi-square analyses (2×2 contingency tables, \( P < 0.05 \)) comparing treatments with control (see Section 2). For HD125, 20 and 23 h treatments were significantly different in all three experiments; for HD11, 23 h treatment was significantly different in one experiment.](image-url)
range from middle logarithmic up to and beyond early stationary phases,\textsuperscript{11,14,19,21} such as Vip3A-like and/or Cry 1I toxin. It is also relevant to consider that narrow peaks of activity (within $\sim$1 h growth time) may be present in a strain’s growth curve, so that extraction and assessment of protein toxicity might be needed at shorter time intervals than those presented in this study.

The temporal analyses of variation in growth (Fig. 1a), protein secretion (Figs 1b and c and Fig. 2) and SN toxicity (Fig. 3) suggest that a fully adequate assessment of the insecticidal potential of isolates in insect feeding assays would depend on the knowledge of their respective physiologies in culture (Figs 1b and 2). An 8 h difference among isolates in terms of entering the log phase can definitely generate confounding effects if SNs collected before the stationary phase are compared (Fig. 1a). Moreover, even if SNs are collected at a time at which all isolates have already reached stationary phase, the isolate-specific variation in the quantitative (Fig. 1b) and qualitative (Fig. 2b) patterns of protein secretion is still high, which may generate distinct toxicity profiles for each strain (Fig. 3). This would be enough to cause misclassification of certain isolates if the time chosen for comparison corresponded to lower levels of their active entomotoxins in the SN, or to possibly unknown types of molecular interaction (repressive or synergistic) that might alter the final toxicity of the SN proteins. The comparative assessment of SNs at later sporulation stages (which are much easier to target in many cultures grown simultaneously) might be a possible alternative to compensate for the physiological variation observed during exponential growth, assuming that the secreted entomotoxin of interest is accumulated up to that point. However, previous knowledge about temporal stability in culture and/or degradation rates of toxins secreted early in SN is certainly required.

The observation that larval mortality (Fig. 3) (and, potentially, reduction in pupa formation) varied during cultures for non-heated SN aliquots suggests that the secretion of proteic entomotoxins (thermolabile fraction) is subjected to an isolate-specific temporal variation in concentration and/or activity. The denaturing polyacrylamide-gradient electrophoretic profiles confirmed such a variation, as various peptides showed differences in presence/absence and intensity of bands among isolates and times (Fig. 2). The presence of peptides with molecular weights consistent with those of Vip3A, Cry 1I, phospholipase-C and exochitinase toxins might help explain the toxicity presented by the corresponding SN (e.g. HD125 after 20 h) (Figs 2 and 3). The rich peptide profile observed for HD125 also presented bands of the expected size for Vip2A, Vip1A and Sip 1A. However, it would be surprising if they were involved in the observed toxicity against fall armyworm (Fig. 3), as they have been reported as being specifically active against coleopteran species.\textsuperscript{11,18}

Although at low levels, the presence of mortality (Fig. 3) and delay in pupation (not shown) for some isolates/times on the SN-$\Delta$ treatments suggest that some proportion of non-proteic (thermostable) toxins (e.g. $\beta$-exotoxins) may coprecipitate with the proteins. Heat treatment appeared to be useful in differentiating the fraction of SN toxicity that is in fact related to proteotoxins, at least when using a simple and cheap method for extraction of secreted proteins, such as (NH$_4$)$_2$SO$_4$ precipitation. However, considering that thermostable toxins tend to be produced more from late exponential growth up to sporulation,\textsuperscript{32} which was a period not fully included in the feeding tests, further work spanning such a period is needed to ensure that the levels of thermostable toxin coprecipitation with proteins are kept at sufficiently low levels.

Results also showed that higher insect toxicity of an isolate SN is not necessarily related to higher protein contents. For instance, while HD125 showed a peak of insect toxicity that coincided with the period of maximal protein secretion, HD11 did not (Figs 1c and 3). This could be explained either by a high concentration of the specific entomotoxins(s) causing death, even in an overall low total protein background, or, alternatively, by possible molecular interactions in the SN that could modulate stability, activation or inhibition of toxic activities.\textsuperscript{33,34} Also, possible temporal variation in entomotoxin gene expression cannot be ruled out.\textsuperscript{9,33} Another relevant aspect is that acute effects (mortality) may not be the only form of toxicity worth assessing.\textsuperscript{25–37} Although not appropriately reproducible (see Section 3), significant reduction in pupa formation was indeed observed for all isolates and most culture times, even when mortality was low or not detected (Fig. 3, data not shown). This suggests that a screening system based also on chronic forms of toxicity may be worth developing for $Bt$ collections.\textsuperscript{25,37} This type of activity can prevent or delay completion of the insect pest life cycle, thereby affecting the establishment of the next larval generation which increases potential damage to the plants. In fact, it is reasonable to assume that the limit separating entomotoxic effects by larval mortality from developmental delays in pupa formation may not be so clear and sharply reproducible. In this context, further experiments assessing whether delay in pupa formation is associated with reduction in average larval weight and/or rate of feeding are certainly warranted.

The results presented here essentially demonstrate that screening procedures on large collections for SN-derived entomotoxins must deal with physiological differences among isolates that will ultimately affect how the selection strategy will be established. Certainly, technical and economical issues are to be pondered in these circumstances, such as potentially significant increases in costs and/or labour. The decision-making process should include various aspects, such as (i) how many isolates can be included in a round of screening, (ii) how long should these isolates be grown for comparison purposes, (iii) how many time points of culture may be assessed to understand an isolate’s physiology, and so increase efficiency of the screening process, (iv) what fraction of the toxins of interest produced during exponential growth remain stable and active in SNs collected after the sporulation stages, (v) how much previous knowledge exists about the testing of isolates that can influence these decisions, (vi) how many new efficient isolates are enough for research purposes, (vii) the degree to which the culture medium pH values and buffering will interfere with the screening process, etc. Moreover, depending on the scheme adopted for the insect rearing system (e.g. maintenance of genetic variability to prevent deleterious inbreeding effects in the egg source population), intrinsic experimental variability is a problem in feeding bioassays that should also be considered,\textsuperscript{14} as it can affect its sensitivity and efficiency in sorting toxic effects among isolates. It is hoped that the information presented in this study be useful in helping to orient other screening strategies for SN-secreted toxins from $Bt$ isolates, mainly for those systems that will depend upon feeding bioassays to accomplish a proper selection scheme for strains or toxins with biocontrol potential.

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