**Bacillus thuringiensis** subsp. **kurstaki** HD1 as a factory to synthesize alkali-labile ChiA74Δsp chitinase inclusions, Cry crystals and spores for applied use

José Eleazar Barboza-Corona1,2*, Jorge Luis Delgadillo-Ángeles1, José Cristóbal Castañeda-Ramírez1, Uriel Eleazar Barboza-Pérez3, Luz Edith Casados-Vázquez1, Dennis K Bideshi4,5 and Ma Cristina del Rincón-Castro1,2

**Abstract**

**Background:** The endochitinase ChiA74 is a soluble secreted enzyme produced by *Bacillus thuringiensis* that synergizes the entomotoxigenecity of Cry proteins that accumulate as intracellular crystalline inclusion during sporulation. The purpose of this study was to produce alkaline-soluble ChiA74Δsp inclusions in *B. thuringiensis*, and to determine its effect on Cry crystal production, sporulation and toxicity to an important agronomical insect, *Manduca sexta*. To this end we deleted the secretion signal peptide-coding sequence of chiA74 (i.e. chiA74Δsp) and expressed it under its native promoter (pEHchiA74Δsp) or strong chimeric sporulation-dependent cytA-p/STAB-SD promoter (pEBchiA74Δsp) in *Escherichia coli*, acrystalliferous *B. thuringiensis* (4Q7) and *B. thuringiensis* HD1.

**Results:** Based on mRNA analyses, up to ~9-fold increase in expression of chiA74Δsp was observed using the cytA-p/STAB-SD promoter. ChiA74Δsp (~70 kDa) formed intracellular inclusions that frequently accumulated at the poles of cells. ChiA74Δsp inclusions were dissolved in alkali and reducing conditions, similar to Cry crystals, and retained its activity in a wide range of pH (5 to 9), but showed a drastic reduction (~70%) at pH 10. Chitinase activity of *E. coli*-pEHchiA74Δsp was ~150 mU/mL, and in *E. coli*-pEBchiA74Δsp, 250 mU/mL. 4Q7-pEBchiA74Δsp and 4Q7-pEHchiA74Δsp had activities of ~127 mU/mL and ~41 mU/mL, respectively. The endochitinase activity in HD1-pEBchiA74Δsp increased 42x when compared to parental HD1 strain. HD1-pEBchiA74Δsp and HD1 harbored typical bipyramidal Cry inclusions, but crystals in the recombinant were ~30% smaller. Additionally, a ~3x increase in the number of viable spores was observed in cultures of the recombinant strain when compared to HD1. Bioassays against first instar larvae of *M. sexta* with spore-crystals of HD1 or spore-crystal-ChiA74Δsp inclusions of HD1-pEB-chiA74Δsp showed LC50s of 67.30 ng/cm² and 41.45 ng/cm², respectively.

**Conclusions:** Alkali-labile ChiA74Δsp inclusion bodies can be synthesized in *E. coli* and *B. thuringiensis* strains. We demonstrated for the first time the applied utility of synthesis of ChiA74Δsp inclusions, Cry crystals and spores in the same sporangium of HD1, a strain used successfully worldwide to control economically significant lepidopteran pests of agriculture. Our findings will allow to us develop strategies to modify expression of ChiA74Δsp while maximizing Cry crystal synthesis in commercial strains of *B. thuringiensis*.

**Keywords:** *Bacillus thuringiensis*, Endochitinase ChiA74, Cry proteins, Inclusion bodies
Background

*Enterobacter cloacae,* *Pseudomonas putida,* and *Pseudomonas fluorescens* are the most successful commercial bioinsecticides used worldwide [3,4], and are composed of a plethora of Cry (crystal) or Cyt (cytolytic) proteins that are synthesized and secreted during vegetative growth that hydrolyze environmental chitin substrates for use as carbon and nitrogen sources. Chitinases are generally produced at a markedly lower level than Cry and Cyt. As such, unlike Cry and Cyt, and perhaps disadvantageous to efficient commercial formulations, chitinases do not naturally accumulate as intracellular inclusions in bacterial cells [9-14].

From an applied perspective, chitinases could be a useful component of *B. thuringiensis*-based biopesticides as they could function to degrade chitin polymers present in the protective midgut peritrophic membrane of insect larvae. In fact, previous studies have demonstrated that the hydrolytic activity of chitinase synergizes the toxicological effects of Cry, presumably by enhancing binding of active toxin ligand to microvillar membrane receptors [11,13-15]. Interestingly, it has been shown that when the chitinase gene of *B. thuringiensis* strain 4.0718 lacking its secretion signal peptide coding sequence was expressed under sporulation-dependent promoters, spherical intracellular inclusion accrued, and when these purified inclusions were mixed with Cry1Ac, an ~1.5x increase in toxicity in against *Spodoptera exigua* and *Helicoverpa armigera* was observed [11]. Similar studies using secreted soluble chitinases have also demonstrated that these enzymes enhance the toxicity of Cry1Ac. For example, the chitinase gene of *Nict利亚tiana tabacum* expressed simultaneously with cry1Ac in an acrystalliferous strain of *B. thuringiensis* using the BtI-BtII promoters showed increases in chitinolytic activity (6x) and toxicity (18x) of the recombinant bacterium against *Helicoverpa armigera* Hubner [14]. These methods of expression of chitinase genes under sporulation-dependent promoters appear to be more robust compared to the synthesis of unstable chimeric protein composed of Chi255 and the C-terminal half (crystallization domain) of Cry1Ac [15].

Recently, we have reported an unprecedented ~300-fold increase in synthesis of ChiA74, an endochitinase native to *B. thuringiensis*, in a recombinant strain of *B. thuringiensis* HD73 [16] when chiA74 was expressed under control of the strong chimeric promoters, cytA-p/STAB-SD, developed by Park et al. (1998) [17]. In this study, we used the wildtype promoter of chiA74 or cytA-p/STAB-SD to express chiA74 lacking the sequence coding for the secretion signal peptide (chiA74sp) in *Escherichia coli*, acrystalliferous *B. thuringiensis* subsp. *israelensis* strain 4Q7, and *B. thuringiensis* subsp. *kurstaki* HD1, a strain used successfully worldwide in agriculture as a biodegradable lepidopteran larvicide. Using this strategy, we were able to produce stable ChiA74sp inclusions in *E. coli*, and also, for the first time, intracellular ChiA74sp inclusion together with Cry crystals and spores in *B. thuringiensis* subsp. *kurstaki* HD1. We demonstrate the utility of the recombinant HD1 strain against larvae *Manduca sexta*. Our results lay a foundation for similar engineering of other commercial strains of *B. thuringiensis*.

Results

ChiA74sp accumulates as inclusion bodies in *Escherichia coli*

When *E. coli* was transformed with the constructs lacking the secretion peptide signal sequence coding for amino acids 1-34, i.e. pEHchiA74sp (native promoter) and pEBchiA74sp (sporulation-dependent cytA-p/STAB-SD promoter) (Figure 1A), recombinant *E. coli*-pEHchiA74sp and *E. coli*-pEBchiA74sp showed activities of 150 and 250 mU/mL, respectively; no activity was observed in the control *E. coli* strain (Figure 1B, panel 2). In *in situ* assays using the 4-MU-(GlcNAc)₃ substrate with proteins fractionated by SDS-PAGE, ChiA74sp was detected by zymograms as a protein of ~70 kDa produced by both recombinant strains (Figure 1B, panel 1), but not in the control strains. To visualize the location of the chitinase in *E. coli*, ChiA74sp was fused to the green fluorescent protein (ChiA74sp-GFP). Fluorescence was observed within the cytoplasm of the cell, confirming the cytoplasmic location of ChiA74sp, the fluorescent chimera frequently accumulated as inclusion bodies at the poles of the cells, as revealed by microscopy (Figure 2B, panels 1, 3, 5). A similar phenomenon has been observed in aging cultures of *E. coli* expressing other heterologous proteins lacking their native secretion signal peptide sequence [18].

ChiA74sp accumulates as intracellular inclusions in *acrystalliferous Bacillus thuringiensis* 4Q7

When pEHchiA74sp and pEBchiA74sp were introduced in 4Q7, the recombinant bacterium produced inclusion bodies in the cytoplasm, readily detected by phase contrast and fluorescence microscopy (Figure 1C; Figure 2B, panels 2, 4). Interestingly, small inclusion bodies dispersed along the cytoplasm appeared to be concentrated at the cell pole before lysis, and were
Figure 1 Expression of ChiA74Δsp in Escherichia coli DH5α and Bacillus thuringiensis 4Q7. (A) Schematic illustration of the strategy used to delete the secretion signal peptide-encoding sequence (shown as a rectangle inside the open reading frame) of chiA74 to generate chiA74Δsp. Two constructs were developed, the first under regulation of wildtype promoter (wp) and the second under control of the strong cytA-p/STAB-SD promoter system. Lollipop indicates the putative transcriptional terminator site. Oligonucleotide sequences used to delete the signal peptide-encoding sequence are shown in Table 1.

(B) Evaluation of endochitinase activity using solubilized intracellular proteins produced in E. coli. Panel 1: Zymogram using 4-MU-(GlcNAc)3 for detection. Lane 1, E. coli-pEHchiA74Δsp; lane 2, without sample; lane 3, E. coli-pEBchiA74Δsp; lane 4, E. coli DH5α. Black arrow indicates the position of ChiA74Δsp in recombinant E. coli strains. Panel 2: (a) E. coli-pEHchiA74Δsp, (b) E. coli-pEBchiA74Δsp. No endochitinase activity was observed with E. coli DH5α. (C) Phase contrast microscopy of recombinant strains of B. thuringiensis. Panel 1, 4Q7; panel 2, 4Q7-pEHchiA74Δsp; panel 3, 4Q7-pEBchiA74Δsp. Black arrows indicate the positions of ChiA74Δsp inclusions. (D) Endochitinase activity determined using solubilized intracellular proteins of recombinant strains of B. thuringiensis. Panel 1: lane 1, 4Q7; lane 2, 4Q7-pEHchiA74Δsp; lane 3, 4Q7-pEBchiA74Δsp. Black arrow indicates the position of ChiA74Δsp in recombinant 4Q7 strains. Panel 2: (a) 4Q7-pEHchiA74Δsp, (b) 4Q7-pEBchiA74Δsp. Activity of recombinant bacteria was normalized with the residual intracellular endochitinase activity of 4Q7.
observed as dark bodies by phase contrast microscopy (Figure 1C, panels 2, 3). This phenomenon was confirmed when ChiA74Δsp-GFP was expressed in 4Q7 (Figure 2B, panel 2). When recombinant B. thuringiensis 4Q7 cells were disrupted and the intracellular proteins dissolved under alkaline conditions and assayed with the

Figure 2 Confirmation of the intracellular location of ChiA74Δsp in Escherichia coli and Bacillus thuringiensis 4Q7 using a chimeric construct with the green fluorescent protein (GFP) gene. (A) Schematic illustration that shows the fusion of chiA74Δsp with gfp. Panel 1, two constructs were developed, under regulation of the chiA74 wildtype promoter (wp) or under control of the cytA-p/STAB-SD system. Lollipop indicates transcriptional terminator. Oligonucleotides used to make chimeric construct with gfp are shown in Table 1. Panel 2, confirmation of chimeric constructs by PCR, primers used for amplification are showed in parenthesis and in Table 1. L, 1 kb (kilobase) DNA Ladder (New England Biolabs); Lane 1, pEHchiA74Δsp-gfp (primers: chiA74-1, chiA74-3); lane 2, pEHchiA74Δsp-gfp (primers: chiA74-1, gfp-3); lane 3, pEHchiA74Δsp-gfp (primers: gfp-1, chiA74-4); lane 4, pEBchiA74Δsp-gfp (primers: cytSTAB-1, chiA74-4); Lane 5, pEBchiA74Δsp-gfp (primers: cytSTAB-1, gfp-3); lane 6, pEBchiA74Δsp-gfp (primers: gfp-1, chiA74-4). (B) Phase contrast (left) and fluorescence micrographs (right) of recombinant strains of E. coli and B. thuringiensis strain 4Q7 expressing the chimeric protein ChiA74Δsp-GFP. Panel 1, E. coli-pEHchiA74Δsp-gfp; panel 2, 4Q7-pEHchiA74Δsp-gfp; panel 3, E. coli-pEBchiA74Δsp-gfp; panel 4, 4Q7-pEBchiA74Δsp-gfp; panel 5, E. coli DH5α; panel 6, 4Q7. Samples of recombinant strains of B. thuringiensis were collected at ~72 h.
chitin-derived fluorogenic substrate, data normalized with the residual activity in 4Q7 showed that 4Q7-pEBchiA74Δsp had an activity of ~127 mU/mL, whereas 4Q7-pEHchiA74Δsp had an activity of ~41 mU/mL. (Figure 1D, panel 2). The higher production of ChiA74Δsp in 4Q7-pEBchiA74Δsp could be attributed to the endochitinase gene expressed using the strong cytA-p/STAB promoter as quantitative PCR showed the relative amount of chiA74Δsp-specific mRNA increased by ~9-, 5-, 3- and 2-fold when compared to expression with the native promoter at 6, 8, 12 and 24 h, respectively. We note that the highest mRNA chiA74Δsp expression was observed at 12 h (Figure 3). In addition, ChiA74Δsp was detected by zymograms in both recombinant strains as a protein of ~70 kDa. Under the time of UV exposure, we did not observe a fluorescence signal produced by native 4Q7 chitinases (Figure 1D, panel 1).

ChiA74Δsp forms stable inclusions in HD1
The initial studies in E. coli and 4Q7 demonstrated that ChiA74Δsp could be stably produced as intracellular inclusions in these bacteria. To further our studies, we transformed HD1 with pEBchiA74Δsp and confirmed the fidelity of the recombinant by PCR using specific oligonucleotides (Table 1) to detect the erythromycin resistance gene (~1 kb) and the endochitinase gene under control of the cytA-p/STAB-SD system (~3 kb); both genes were not detected in wildtype HD1 or pBluescript KS(+) (Stratagene) which were used as negative controls (Figure 4F). When HD1-pEBchiA74Δsp was observed by phase contrast microscopy, ChiA74Δsp inclusion bodies, most commonly occurring at the sporangium pole, could be easily distinguished from the native bipyramidal crystals and endospores (Figure 4A,B,C). Fluorescence microscopy of ChiA74Δsp-GFP confirmed the intracellular location of the inclusion (Figure 4D,E).

The effect of ChiA74Δsp synthesis on Cry crystal size in HD1-pEBchiA74Δsp was also determined. A reduction by ~33% in area of the Cry crystalline inclusion was observed in the recombinant producing ChiA74Δsp when compared to crystals produced by wildtype HD1 (Table 2). Although the area (two-dimensional surface) determination is not suggestive of volume (three-dimensional space) to determine the yield of Cry proteins, the decrease in the crystal area correlated well with the reduction in the relative amount of Cry1 (~133 kDa)/Cry2Aa (~65 kDa) proteins detected by SDS-PAGE (Figure 4G). In addition, a band corresponding to endochitinase ChiA74Δsp (~70 kDa), and other smaller bands which could correspond to endochitinase degradation, were observed in the recombinant but not in wildtype HD1, as confirmed by zymogram analyses (Figure 4H). The chitinase activity was markedly increased (~42-fold) in HD1-pEBchiA74Δsp when compared with HD1, respectively, ~127 mU/mL and 3 mU/mL (Table 2). Moreover, we determined the activity of the recombinant chitinase in a range of pH typically observed in lepidopteran larval midgut (~ pH 8–11). The enzyme retained its activity at a range from pH 5 to 9, but it was reduced drastically to ~70% at pH 10 (Figure 5). We also note that at
The use of bacterial chitinases could be of significance in Bacillus thuringiensis-based biocontrol efforts because they synergize insecticidal Cry proteins produced by strains of this species [19-23]. Although increases in synthesis of extracellular chitinases in B. thuringiensis has been accomplished using various expression systems, the practical problem regarding the likely instability of potential mixtures of spore-crystal-soluble chitinase formulations remains to be resolved [9,16]. Ideally, the production of physically stable, but biochemically (alkaline) labile, inclusions of chitinase and Cry crystals in the same cell could alleviate this concern. Hu et al. [11] successfully produced chitinase lacking its secretion signal peptide sequence as inclusions in an acrystalliferous B. thuringiensis strain. However, the concern was not resolved as chitinase inclusions and Cry crystals were synthesized in different bacterial strains of which preparations must be mixed for bioassays, or for prospective commercial formulations. In addition, their work as designed could not address the effect of chitinase synthesis on Cry crystal production and viable spore count of recombinant B. thuringiensis, two factors that must be optimized for potential applied and commercial consideration.

In the present study, we demonstrated that by deleting the secretion signal peptide sequence of ChiA74 (ChiA74Δsp), stable occluded ChiA74Δsp could be produced in different bacteria. First, we transformed E. coli with the constructs to produce sufficient recombinant plasmid DNA to transform B. thuringiensis. To our surprise we observed the formation of small inclusion bodies at the poles of E. coli and demonstrated they were composed of ChiA74Δsp. To our knowledge it is the first report that chitinase inclusion bodies can be produced in E. coli. We note that the synthesis of ChiA74Δsp as stable inclusions in E. coli could have biotechnological value, as it could be mass-produced and easily purified using an organism “generally recognized as safe” (e.g. E. coli K12) for applied use, such as for generating chitin-derived oligosaccharides with pharmaceutical and/or food preservation properties [10].

Our major objective in this work was to produce, for first time, ChiA74Δsp inclusions together with insecticidal crystals and spores in the same cell, study its cellular effect and determine the recombinant’s toxicity to an important agricultural insect such as Manduca sexta larvae. We observed the formation of ChiA74Δsp inclusions in the acrystalliferous B. thuringiensis 4Q7, and like in E. coli, they accumulated at the poles. Increased chitinase synthesis was observed when the endochitinase gene was expressed using the strong cytA-p/STAB promoter system developed by Park et al. (1998) (17), compared to regulation by its native promoter, and most likely was a result of increased chiA74Δsp mRNAs, as demonstrated by qPCR. Interestingly, when we transformed the acrystalliferous strain B. thuringiensis HD1 with the endochitinase gene chiA74Δsp regulated by cytA-p/STAB, we observed two important changes in the recombinant strain: (i) a reduction in the crystal size (i.e. less Cry production) and (ii) a 3-fold increase in the number of viable spores. With regards to crystals, we observed an ~33% decrease in the Cry crystals area (Figure 4A,B,C, Table 2) similar to previous reports [9,16], which correlated well

| Primer | Sequence* |
|--------|-----------|
| chiA74-13 | 5′-TCCCGCGAGTAATCCAAAGCGCAAGATGTTTGC-3′ |
| chiA74-12 | 5′-TCCCGCGGGTTTCCTCTTCTAAATTAAAGATATTTAAGGCG-3′ |
| gfp-1 | 5′-ATGCTAGCAAAAAGGAGAAGACTT-3′ |
| gfp-3 | 5′-GGTGAATTTTATTTTCGATTTC-3′ |
| chiA74-C | 5′-GGTGAATTTTATTTTCGATTTC-3′ |
| chiA74-B | 5′-GTCTCTGCTAATGACGGCATTTAAAAG-3′ |
| cyt-STAB-1 | 5′-GCCAATTCATTTTCGATTTC-3′ |
| chiA74-3 | 5′-AACTGCAAGGCAAGGCTTCCCTAACAAGGTGACTAC-3′ |
| ery-1 | 5′-AAAACACTGCAGCAGTTAACAGGATGTTGTTGATATTGC-3′ |
| ery-2 | 5′-ATAAGAATGGCCTGCGCCCGGTAGCCGCTAGGGACC-3′ |

*Artificial start ATG codon (bold type) used to initiate the chitinase translation, and the restriction endonuclease cleavage sites SacII, BglII, PstI and NotI (italics) are shown.

The same period of growth (72 h) in nutrient broth, the viable spore count for the recombinant was ~3-fold greater when compared with HD1 (Table 2).

Bioassays
Spore-Cry crystal mixtures of HD-1 and spore-Cry crystal-ChiA74Δsp inclusion mixtures of HD1-pEBchiA74Δsp were assayed against first instar larvae of the tobacco hornworm (Manduca sexta). The LC50 for HD1 was 67.30 ng/cm² diet and 41.45 ng/cm² diet for HD1-pEBchiA74Δsp, representing an apparent 1.6x enhancement in toxicity of the recombinant strain. However, we did not detect a significant difference in the LC50s as there was an overlap between the upper fiducial limit of the recombinant strain’s LC50 and the lower fiducial limit of the wildtype LC50. A difference of 25.84 ng/cm² diet between both LC50s, only showed that the recombinant strain required lower concentration than the wildtype to kill 50% of the larvae (Table 3). In addition, no toxicity was observed (0% mortality) against M. sexta using spores-ChiA74Δsp inclusion mixtures synthesized in 4Q7 (data not shown).

Discussion
The use of bacterial chitinases could be of significance in Bacillus thuringiensis-based biocontrol efforts because they synergize insecticidal Cry proteins produced by strains of this species [19-23]. Although increases in synthesis of extracellular chitinases in B. thuringiensis has been accomplished using various expression systems, the practical problem regarding the likely instability of potential mixtures of spore-crystal-soluble chitinase formulations

---

Table 1 Primers used for PCR construction and amplification of chiA74Δsp and chiA74Δsp-gfp

| Primer | Sequence* |
|--------|-----------|
| chiA74-13 | 5′-TCCCGCGAGTAATCCAAAGCGCAAGATGTTTGC-3′ |
| chiA74-12 | 5′-TCCCGCGGGTTTCCTCTTCTAAATTAAAGATATTTAAGGCG-3′ |
| gfp-1 | 5′-ATGCTAGCAAAAAGGAGAAGACTT-3′ |
| gfp-3 | 5′-GGTGAATTTTATTTTCGATTTC-3′ |
| chiA74-C | 5′-GGTGAATTTTATTTTCGATTTC-3′ |
| chiA74-B | 5′-GTCTCTGCTAATGACGGCATTTAAAAG-3′ |
| cyt-STAB-1 | 5′-GCCAATTCATTTTCGATTTC-3′ |
| chiA74-3 | 5′-AACTGCAAGGCAAGGCTTCCCTAACAAGGTGACTAC-3′ |
| ery-1 | 5′-AAAACACTGCAGCAGTTAACAGGATGTTGTTGATATTGC-3′ |
| ery-2 | 5′-ATAAGAATGGCCTGCGCCCGGTAGCCGCTAGGGACC-3′ |

*Artificial start ATG codon (bold type) used to initiate the chitinase translation, and the restriction endonuclease cleavage sites SacII, BglII, PstI and NotI (italics) are shown.
with a decrease in Cry protein synthesis as detected by SDS-PAGE (Figure 4G). The increase in spore count was not expected based on results of a previous study where the opposite occurred following expression of heterologous chitinase (secreted) in B. thuringiensis [16]. Although we do not have supporting experimental evidence, it is possible that the synthesis of two kind of proteins, Cry and ChiA74, whose gene expression is controlled by two strong sporulation-dependent promoters (BtI-BtII, cyt-p/STAB) incur a more rapid depletion of nutrients thereby inducing sporulation. For example, it is known that the activation of Spo0A, a master regulator for entry into sporulation in B. subtilis, is induced in response to nutrient limitation [24,25].

As suggested previously, the advantage of producing ChiA74Δsp inclusions in HD1 allow the direct use of spore-Cry crystals-ChiA74Δsp mixtures from a single source in bioassays, rather than mixing preparations...
from different strains as reported previously [11]. We were successful in engineering a recombinant HD1 strain producing ChiA74Δsp inclusions during sporulation that had a 42-fold increase in chitinase activity. Despite the marked increase in chitinase activity, only an apparent 1.6-fold increase in toxicity was observed against *M. sexta* first instar larvae. Similar results (1.5-fold increase) have been observed with mixtures of different recombinant strains producing chitinase inclusions and Cry1Ac against *S. exigua* and *H. armigera* [11]. It is likely that the decrease in Cry crystal synthesis in the recombinant strain lowered the expectations of several-fold increases in insecticidal activity of the recombinant. In addition, and probably of more significance, is the marked decrease (~70%) in enzymatic activity of preparations of ChiA74Δsp inclusions solubilized at pH 10 (Figure 5). It is worth noting that lepidopteran midguts normally show pH gradients from anterior to posterior, and from the lumen to epithelial microvilli. The midgut of *M. sexta* larva ranges in pH from ~10–11 [26]. Although it is evident that the lower LC₅₀ of the recombinant strain is a consequence of an increase chitinase production with the compensating decrease in Cry crystal proteins, our results suggest that more “balanced” expression of both cry and chiA74Δsp could result in optimal production of these proteins conducive to an efficacious biopesticide. In summary, we have produced ChiA74Δsp inclusion in HD1 and the recombinant showed an apparent increased activity against first instar *M. sexta* larvae. Our future studies include producing ChiA74Δsp inclusions in other lepidopteran-, coleopteran- and dipteran-specific strains of *B. thuringiensis* for bioassays against a wide variety of insect larvae. Finally, we are also in the process of using molecular strategies to modify expression of ChiA74Δsp, while at the same time maximizing the production of endogenous Cry proteins to develop highly efficacious strains of *B. thuringiensis* for applied use.

**Conclusions**

Inclusions of ChiA74Δsp can be produced in *E. coli* and *B. thuringiensis* strains. We show for the first time, the ability to synthesize ChiA74Δsp inclusions, insecticidal Cry crystals and spores in the same sporangium. We observed that the production of ChiA74Δsp inclusions affect the crystal size and sporulation in *B. thuringiensis* subsp. *kurstaki* HD1. The data reported in this study lay a foundation for developing strategies to modify expression of chiA74Δsp while maximizing the production of Cry proteins.

**Material and methods**

**Bacterial strains and plasmids**

Plasmids pEHchiA74 and pEBchiA74 harbor the chiA74 under the control of, respectively, the wild promoter (wp) and the 660-bp strong chimeric sporulation-dependent pcytA-p/STAB-SD promoter developed by Park et al. (1998) [17]. The wildtype Shine-Dalgarno (wSD) and transcription terminator (chiA74tt) sequences were retained in all constructs [9,16]. These plasmids (pEHchiA74 and pEBchiA74) were used for deleting the signal peptide-encoding sequence of ChiA74 to obtain ChiA74Δsp.

### Table 2 Endochitinase activity (U)*, crystal area and viable spores of wildtype and recombinant strains of *Bacillus thuringiensis*

| Strain            | HD1-pEBchiA74Δsp | HD1   |
|-------------------|------------------|-------|
| µU/mL (±SD)       | 127 (± 20)*      | 3.0 (± 0.2)* |
| Ratio             | 42.0             | 1.0   |
| Crystal area (µm²) (±SD) | 0.86 (± 0.16)* | 1.28 (± 0.19)* |
| Ratio             | 1.0              | 1.5   |
| Spores/mL x 10⁷ (±SD) | 8.35 (± 0.57)* | 2.95 (± 0.21)* |
| Ratio             | 2.83             | 1.0   |

*One unit (U) was defined as the amount of enzyme required to release 1 µmol of 4-methylumbelliferone in 1 h.

**Values with different letter (a, b) in the same row are significantly different as determined by Tukey’s multiple range test (p < 0.05).**

### Table 3 Statistical parameters for estimating the LC₅₀ of strains of *B. thuringiensis* against tobacco hornworm *Manduca sexta*

| Strain            | LC₅₀ (±95% CI) | Slope | X² | Mortality |
|-------------------|---------------|-------|----|-----------|
| HD1               | 67.29 (47.18-95.98) | 1.70  | 0.90 | 0         |
| HD1-pEBchiA74Δsp  | 41.45 (28.33-60.64) | 1.50  | 0.17 | 0         |

Values are shown in nanograms per cm² of spore-crystal mixture or spore-crystal-ChiA74Δsp inclusion bodies mixture for HD1 and HD1-pEBchiA74Δsp, respectively, and represent 5-days mortality as determined by Probit analysis. Fiducial limits are indicated in parenthesis.
acrystalliferous strain of Carlsbad CA, USA) and then used for transforming the

pSTAB, a pHT3101-derived vector containing cyt1A-

resistance gene markers [27].

The shuttle vectors used to transform the different

trol arabinose (LB) medium with ampicillin (100

μg L-1)(10). 4Q7 and HD1 competent cells were prepared as described previously [28]. Approximately 3 μg of the recombinant plasmids were mixed with 300 μl of competent cell sus-
pension, held on ice for 10 min followed by electroporation using a BTX ECM630 electro cell manipulator (San Diego CA, USA) set at 2.3 kV, 475 Ω and 25 μF. After the pulse, the suspension was added to 3 ml of brain heart in-
fusion (BHI) (Bioxon México) and incubated with gentle shaking for 1 h at 37°C. Transformants were selected on BHI supplemented with 25 μg mL^-1 of erythromycin.

Phase contrast and fluorescence microscopy

E. coli, 4Q7 and HD1 were cultivated in LB or nutrient broth at 37°C or 28°C (200 rpm), respectively. Samples were taken at different times and monitored by phase contrast and fluorescence microscopy. Data were obtained using an Axio Imager A1 Zeiss microscope with the filter set at 09, an excitation of 450–490 nm, and an emission of 515 nm. Crystal area was estimated using the AxioVision LE program (Carl Zeiss Microscopy, Göttingen Germany).

Evaluation of the chitinase activity

To determine the level of endochitinase activity in prepa-

rations of E. coli-pEHchiA74Δsp, E. coli-pEBchiA74Δsp and 4Q7-pEBchiA74Δsp, 4Q7-pEBchiA74Δsp, bacteria were cultivated in LB with ampicillin (100 μg/ml) at 37°C, 200 rpm, or in nutrient broth with erythromycin (25 μg/ml) at 28°C, 200 rpm [16], respectively. Controls (E. coli and 4Q7) were grown without antibiotics. Cultures were centrifuged, washed three times with distilled water and resuspended in 100 mM phosphate buffer (pH 7.0). Cells were sonicated three times, 15 s each, at an amplitude of 40 Hz in a 20 kHz ultrasonic processor (Sonics and Materials, Inc). Samples were centrifuged and the pellets mixed with solubilization buffer (30 mM Na2CO3, 0.2% β-ME, 1 mM phenyl-
methylsulfonyl fluoride, pH 10–11 [11]. Suspensions were incubated at 37°C with gentle agitation for 40 min, centrifuged, and supernatants were assayed with
the fluorogenic substrate 4-MU-(GlcNAc)₃ at pH 6.8, in a Glomax Multi Jr. Detection System (Promega, Sunnyvale CA, USA), as previously described [29]. To determine chitinase activity of HD1 and HD1-pEBchiA74Δsp, bacteria were grown in 75 mL nutrient broth with or without erythromycin, respectively, and incubated at 28°C, 200 rpm to autolysis (~72 hr). To compare activity of the recombinant versus wildtype strain, 1 mL of each culture was centrifuged and the pellets were washed three times with distilled water and then resuspended in 150 μL of solubilization buffer. Samples were incubated at 37°C with gentle agitation for 40 min, centrifuged, and supernatants were assayed with the fluorogenic substrate 4-MU-(GlcNAc)₃ at pH 6.8. In addition, activity at different pH of the alkaline-solubilized recombinant chitinase was determined. Approximately 74 mL of the remaining culture was centrifuged, washed with distilled water and resuspended in 5 mL of solubilization buffer. The enzymatic activity of concentrated ChiA74Δsp at a pH range of 4–10 was evaluated with the tetrameric fluorogenic derivative using a reaction buffer containing sodium acetate, MES [2 (N-morpholino) ethane sulfonic acid], NaH₂PO₄, Trizma base [Tris(hydroxymethyl) aminomethane] and glycine, with a final concentration of 15 mM for each component.

In addition, dissolved ChiA74Δsp samples were fractionated in a 12% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Afterwards proteins were renatured by removing SDS and 2-mercaptoethanol with casein-EDTA wash buffer (1% casein, 2 mM EDTA, 40 mM Tris–HCl, pH 9). Detection of chitinase activity was determined using 4-MU-(GlcNAc)₃, as previously described [29].

Effect on viable spores count
Bacteria were grown in nutrient broth for 3 days at 28°C, 200 rpm. Then 100 μL of autolysed cultures were incubated at 60°C for 20 min to destroy remaining vegetative cells [16]. After serial dilution (10⁻⁵–10⁻⁶), suspensions were plated on nutrient agar with or without erythromycin and incubated at 28°C for 24 h to determine the number of viable spores. Data were analyzed with the ANOVA program (StatSoft Inc.).

Quantitative PCR (qPCR)
Total mRNA was obtained from 4Q7-pEHchiA74Δsp-gfp and 4Q7-pEBchiA74Δsp-gfp. One mL of each bacterial culture was harvested periodically from 2 h to 96 h, centrifuged and cells were resuspended in 1 mL of Trizol (Invitrogen, Carlsbad CA, USA). Samples were sonicated 15 s in an ultrasonic processor (Sonics and Materials, Inc), and RNA extraction was performed according to manufacturer’s protocol (Invitrogen, Carlsbad CA, USA). Total RNA was resuspended in 30 μL of double distilled water and DNA contamination was eliminated using DNaseI (Jena Bioscience, Jena Germany). Then 1 μg of total RNA was used to synthesize cDNA using the iScript cDNA synthesis kit according to the manufacturer’s instruction (Bio-Rad, Hercules CA, USA). For quantitative PCR, specific primers were used to amplify the erythromycin and green fluorescent protein gene (gfp). The erythromycin gene was used as internal control to normalize the RNA. As the chitinase gene in 4Q7 is amplified with the specific primer of chiA74Δsp (data not shown), the gfp was employed to determine the relative amount of chiA74Δsp mRNA in the recombinant bacteria because this gene was fused to the chiA74Δsp. Quantitative PCR was carried out in the CFX connect Real time system (Bio-Rad, Hercules CA, USA). Reaction mixture contained 5 μL of SYBR green master mix, 0.4 mM of each primer and 40 μg/mL of total transcribed RNA. Thermal cycling conditions were: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 55°C for 60 s. This was followed by a melting curve program of 65 to 95°C with a heating rate of 0.5°C per second. Data were analyzed by relative quantification using the ΔC_T method (Bio-Rad, Hercules CA, USA).

Bioassays
Manduca sexta (Lepidoptera: Sphingidae) colonies were maintained on artificial diet [30] under laboratory conditions at 28 ± 2°C and 70 ± 10% relative humidity, under a 16:8 (light:dark) photoperiod. Strains were cultured in nutrient broth at 28°C, 200 rpm. Then sporulated and autolysed cultures were centrifuged and supernatants were discarded to eliminate secreted molecules such as protease, endogenous chitinases and putative Vip proteins. Pellets (spore-Cry crystal mixtures of HD-1 and spore-Cry crystal-ChiA74Δsp inclusion mixtures of HD1-pEBchiA74Δsp) were washed three times with distilled water, lyophilized and powders were used for bioassays. Six different preparations of HD1 and HD1-pEBchiA74Δsp, and a tap water negative control, were assayed in triplicate. A constant volume of the sample dilution (250 μl) was applied onto the surface of diet contained in Petri dishes (area 60 cm²). Ten first instar larvae were added to each Petri dish and mortality was recorded after five days of incubation under laboratory conditions. The mean concentration at which 50% (LC₅₀) of the larvae died was estimated by Probit analysis [31].

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JEBC designed the experimental setup, obtained financial support, initiated the project, analyzed results and wrote the manuscript. JEBC, JLD, JCCR, UEBP, LECV and MCRC performed the different experiments. DKB and MCRC helped to design the experimental setup and in manuscript preparation. All authors read and approved the final manuscript.
Acknowledgements
This research was supported by Grant SEP-CONACyT (156682) México to J. E. Barboza-Corona. J. L. Delgadillo-Ángeles and L. E. Casados-Vázquez are a graduate student and a Postdoctoral researcher, respectively, and are supported by CONACyT, México. We thank Rubén Salcedo-Hernández from Universidad de Guanajuato and Jorge E. Ibarra, Javier Luévano-Borrel from CINVESTAV México, for their technical support during this study.

Author details
1 Universidad de Guanajuato Campus Irapuato-Salamanca, División Ciencias de la Vida, Posgrado en Biotecnologías, Irapuato, Guanajuato 36500, México.
2 Universidad de Guanajuato Campus Irapuato-Salamanca, División Ciencias de la Vida, Departamento de Alimentos, Irapuato, Guanajuato 36500, México.
3 Tecnológico de Monterrey Campus Querétaro, Epigmenio González 500 Fracc., San Pablo Querétaro, Querétaro 76130, México. 4 Department of Natural and Mathematical Sciences, California Baptist University, 8432 Magnolia Avenue, Riverside California 92504, USA. 5 Department of Entomology, University of California, Riverside California 92521, USA.

Received: 20 November 2013 Accepted: 20 January 2014

References
1. Park H-W, Federci BA, Sakano Y: Insecticidal protein crystals of Bacillus thuringiensis. In Microbiology monographs: inclusions in prokaryotes, Volume 1. Edited by Shively JM, Heidelberg, Germany: Springer-Verlag, 2006:195–236.
2. Wu D, Federci BA: A 20-kilodalton protein preserves cell viability and promotes Cry1A crystal formation during sporulation in Bacillus thuringiensis. J Bacteriol 1993, 175:276–2528.
3. Federci BA: Insecticidal bacteria: an overwhelming success for insect invertebrate pathology. J Invertebr Pathol 2003, 89:30–38.
4. Sanahujia G, Banakar R, Tywmy RM, Capel T, Christou P: Bacillus thuringiensis: a century of research, development and commercial application. Plant Biotechnol J 2011, 9:283–300.
5. Aronson A: Sporulation and d-endotoxin synthesis by Bacillus thuringiensis. Cell Mol Life Sci 2002, 59:417–425.
6. Bietlot HPL, Vishmulhatla J, Carey PR, Pozsgay M, Kaplan H: Efficient synthesis of the 60-kilodalton endochitinase ChiA74 in Escherichia coli. Curr Microbiol 2007, 56:442–446.
7. Smirnoff WA: Regulation of the expression of chitinase genes in Bacillus subtilis. J Bacteriol 1971, 106:539–549.
8. Barboza-Corona JE, Ortíz-Rodríguez T, de la Fuente-Salcido N, Ibarra J, Bideshi DK, Salcedo-Herrández R: Hyperproduction of chitinase influences crystal toxin synthesis and sporulation of Bacillus thuringiensis. Antonie Van Leeuwenhoek 2009, 96:31–42.
9. Park HW, Ge B, Bauer LS, Federci BA: Optimization of Cry3A yields in Bacillus thuringiensis by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. Appl Environ Microbiol 1998, 64:3932–3938.
10. Coquell AN, Jacob JP, Primet M, Demarez A, Dimiccoli M, Julou T, Moisan L, Lindner AB, Berry H: Localization of protein aggregation in Escherichia coli is governed by diffusion and nuclear macromolecular crowding effect. PLoS Comput Biol 2013, 9:e1003398.
11. Kuzu SB, Güvenmez HK, Denizci AA: Weight of Cry1A protein encoded by orf2 in the cry1A operon of Bacillus thuringiensis subsp. kurstaki HD1 as a factory to synthesize alkali-labile ChiA74 protein. Biochem J 2011, 437:365–375.
12. Cai Y, Yan J, Hu X, Han B, Zhiming Yuan Z: Improving the insecticidal activity against resistant Culex quinquefasciatus mosquitoes by expression of chitinase gene chaI in Bacillus sphaericus. Appl Environ Microbiol 2007, 73:7744–7746.
13. Ozgen A, Sezen K, Demir I, Demirbag I, Nalcigolcu R: Molecular characterization of chitinase genes from a local isolate of Seratia marcescens and their contribution to the insecticidal activity of Bacillus thuringiensis strains. Curr Microbiol 2013, 67:400–504.
14. Smerdoff WA: Effect of chitinase on the action of Bacillus thuringiensis. Can Entomol 1971, 102:1829–1831.
15. Smirnoff WA: Improved expression of Cry1A genes in C. elegans by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. Appl Environ Microbiol 2013, 13:355–375.
16. Barboza-Corona JE, Ortíz-Rodríguez T, de la Fuente-Salcido N, Ibarra J, Bideshi DK, Salcedo-Herrández R: Hyperproduction of chitinase influences crystal toxin synthesis and sporulation of Bacillus thuringiensis. Antonie Van Leeuwenhoek 2009, 96:31–42.
17. Park HW, Ge B, Bauer LS, Federci BA: Optimization of Cry3A yields in Bacillus thuringiensis by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. Appl Environ Microbiol 1998, 64:3932–3938.
18. Coquell AN, Jacob JP, Primet M, Demarez A, Dimiccoli M, Julou T, Moisan L, Lindner AB, Berry H: Localization of protein aggregation in Escherichia coli is governed by diffusion and nuclear macromolecular crowding effect. PLoS Comput Biol 2013, 9:e1003398.
19. Buetiott HPL, Vishmulhatla J, Carey PR, Pozsgay M, Kaplan H: Characterization of the cysteine residues and disulfide linkages in the protein crystal of Bacillus thuringiensis. Biochem J 1999, 267:309–315.
20. Chan L, Grant R, Aronson A: Regulation of the packaging of Bacillus thuringiensis d-endotoxin into inclusions. Appl Environ Microbiol 2001, 67:303–5036.
21. Schneip E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH: Bacillus thuringiensis and its pesticidal crystals proteins. Microbiol Mol Biol Rev 1998, 62:757–806.
22. Casque-Arroyo G, Bideshi D, Salcedo-Hernández R, Barboza-Corona JE: Development of a recombinant strain of Bacillus thuringiensis subsp. kurstaki HD-73 that produces the endochitinase ChaI74. Antonie Van Leeuwenhoek 2009, 92:1–9.
23. Castroneda-Ramírez C, De la Fuente-Salcido NM, Salcedo-Hernández R, León-Galván F, Bideshi DK, Barboza-Corona JE: High-level synthesis of endochitinase ChaI74 in Escherichia coli K12 and its promising potential for use in biotechnology. Folia Microbiologica 2013, 58:455–462.
24. Hu SB, Liu P, Ding XZ, Yan L, Sun YJ, Zhang YM, Li WP, Xiao LQ: Efficient constitutive expression of chitinase in the mother cell of Bacillus thuringiensis and its potential to enhance the toxicity of Cry1A protein. Appl Microbiol Biotechnol 2009, 82:1157–1167.
25. Kuzu SB, Güvenmez HK, Denizci AA: Production of a thermostable and alkaline chitinase by Bacillus thuringiensis subsp. kurstaki strain HBK-51. Biotechnol Res Int 2012, Article ID 134989. doi:10.1155/2012/134989.
26. Thamthaihanl S, Moar WJ, Miller ME, Panbangred W: Improving the insecticidal activity of Bacillus thuringiensis subsp. aizawai exigua by chromosomal expression of a chitinase gene. Appl Microbiol Biotechnol 2004, 68:185–192.
27. Ding X, Luo J, Gao B, Sun X, Zhang Y: Improving the insecticidal activity by expression of a recombinant cry1A gene with chitinase-encoding gene in acrylation-resistant Bacillus thuringiensis. Curr Microbiol 2008, 56:442–446.
28. Driss F, Rous S, Azouz H, Tounsi S, Zouari N, Jaoua S: Integration of a recombinant chitinase into Bacillus thuringiensis parasporal insecticidal crystal. Curr Microbiol 2011, 62:281–288.

Cite this article as: Barboza-Corona et al.: Bacillus thuringiensis subsp. kurstaki HD1 as a factory to synthesize alkali-labile ChaI74asp chitinase inclusions, Cry crystals and spores for applied use. Microbial Cell Factories 2014 13:15.