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Biochemical characterization of two chitinases from *Bacillus cereus sensu lato* B25 with antifungal activity against *Fusarium verticillioides* P03

Estefanía Morales-Ruiz[1,†], Ricardo Priego-Rivera[1,†], Alejandro Miguel Figueroa-López[2], Jesús Eduardo Cazares-Álvarez[1] and Ignacio E. Maldonado-Mendoza[1,*,‡]

[1]Departamento de Biotecnología Agrícola, CIIDIR Unidad Sinaloa, Instituto Politécnico Nacional, Boulevard Juan de Dios Bátiz Paredes 250, 81101, Col. San Joachin, Guasave, Sinaloa, Mexico and [2]Departamento de Biotecnología y Ciencias Alimentarias, Instituto Tecnológico de Sonora, 5 de Febrero 818 Sur, Col. Centro, 85000, Ciudad Obregón, Sonora, Mexico

*Corresponding author: Departamento de Biotecnología Agrícola, CIIDIR Unidad Sinaloa,Instituto Politécnico Nacional, Boulevard Juan de Dios Bátiz Paredes No. 250, Col. San Joachin, 81101, Guasave, Sinaloa, México. E-mail: imaldona@ipn.mx

One sentence summary: *Bacillus cereus sensu lato* strain B25 produces two chitinases: ChiA with endo- and ChiB with endo-/exo-chitinase activity, both inhibiting conidial germination of the maize fungal pathogen *Fusarium verticillioides*.

†These authors contributed equally to this work

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‡Ignacio E. Maldonado-Mendoza, http://orcid.org/0000-0001-9952-1508

ABSTRACT

Bacterial chitinases are a subject of intense scientific research due to their biotechnological applications, particularly their use as biological pesticides against phytopathogenic fungi as a green alternative to avoid the use of synthetic pesticides. *Bacillus cereus sensu lato* B25 is a rhizospheric bacterium that is a proven antagonist of *Fusarium verticillioides,* a major fungal pathogen of maize. This bacterium produces two chitinases that degrade the fungal cell wall and inhibit its growth. In this work, we used a heterologous expression system to purify both enzymes to investigate their biochemical traits in terms of K_m, V_{max}, optimal pH and temperature. ChiA and ChiB work as exochitinases, but ChiB exhibited a dual substrate activity and it is also an endochitinase. In this work, the direct addition of these chitinases inhibited fungal conidial germination and therefore they may play a major role in the antagonism against *F. verticillioides*.

Keywords: chitinase; biocontrol; rhizosphere; maize; phytopathogen

INTRODUCTION

The global population is expected to increase by 30% by 2050, with most of the expansion occurring in developing regions (FAO 2017). Such growth will require at least a 50% increase in food production, which could be achieved by any of the following strategies: (i) expanding the area dedicated for agriculture, (ii) intensifying the use of fertilizers, (iii) using improved seeds or (iv) minimizing losses from synthetic pesticide use (Montesinos 2003).

Regarding the use of synthetic pesticides, due to the pathogen resistance as well as their non-targeting effect, there is great interest in the biocontrol of soil-borne pathogens using

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antagonistic microbes as biological pesticides (Montesinos 2003; Singh and Arya 2019).

One such approach involves rhizobacteria, which are known to have both direct and indirect beneficial effects on plants. Direct effects include solubilizing nutrients, fixing nitrogen and producing plant-growth regulators, whereas the indirect effects involve controlling infections by means of producing extracellular enzymes such as chitinases, which degrade fungal and insect chitin (Bhattacharyya and Jha 2012; Singh and Arya 2019). Therefore, some rhizobacteria can be used as a strategy to increase food production by improving plant growth and fighting fungal infections.

Many rhizobacteria from the genera *Pseudomonas*, *Bacillus*, *Erwinia* and *Streptomyces* have a biocontrol effect on pathogenic fungi (Whipps 2001; Haas and Défago 2005; Banerjee and Mandal 2019). Among these, chitinase-producing bacteria stand out in particular, since chitin is a major component of the fungal cell wall (Bhattacharya, Nagpure and Gupta 2007).

Chitin, the second most abundant polysaccharide on the planet after cellulose, is comprised of a long chain of β-1,4-linked polymers of *N*-acetyl-D-glucosamine (GlcNAc), and is the primary component of cell walls and other structural features in fungi, arthropods and other animals (Banerjee and Mandal 2019). Interestingly, chitin multimers (chitotetraose, chitotriose and chitobiose) have broad biotechnological applications (Shahidi and Abuzaytoun 2005). Since chemical degradation of chitin on an industrial scale involves the use of strong acids with low yields and high costs, enzymatic chitin degradation is an interesting alternative approach (Shahidi and Abuzaytoun 2005; Rathore and Gupta 2015).

Chitinases are glycosyl hydrolases that degrade chitin by hydrolyzing its β-1,4 bond (Stoykov, Pavlov and Krastanov 2015). These enzymes are produced by some plants, insects, vertebrates and microorganisms and used for different functions. It has been proposed that bacteria synthesize chitinases in order to obtain nutrients from chitin-containing macromolecules, as well as for use in parasitism and pathogenesis (Adrangi and Faramarzi 2013; Frederiksen *et al.* 2013).

Chitinases are classified into two categories: endochitinases and exochitinases (Singh and Arya 2019). Endochitinases hydrolyze chitin at random internal sites along the polymeric chain, generating soluble low-mass glucosamine multimers (chitotriose, chitobiose and diacetylchitobiose). Exochitinases are further divided in two categories, chitobiosidases and N-acetylglucosaminidases. Chitobiosidases successively release diacetylchitobiose residues from the nonreducing end of the chain, whereas N-acetylglucosaminidases hydrolyze the oligomers released by endochitinases to generate GlcNAc (Rathore and Gupta 2015). Based on their sequence, bacterial chitinases are grouped into families 18, 19 or 20 of the glycosyl hydrolases (Yan and Fong 2015).

Bacillus spp. are known to produce up to four types of chitinases (Rathore and Gupta 2015). The rhizospheric *B. cereus sensu lato* B25 strain has already been characterized in terms of its plant-growth promotion and biocontrol traits. This strain was selected from among 11 520 bacterial isolates from northern Sinaloa in Mexico, based on its ability to reduce *Fusarium verticillioides* (*Fv*) disease severity in maize (Lizárraga-Sánchez *et al.* 2015; Figueroa-López *et al.* 2016). *Fv* causes stalk, ear and root rot in maize, and produces mycotoxins that are toxic to human and animals. Furthermore, *Fv* is the most predominant species isolated in maize fields in northern Sinaloa, Mexico (Leyva-Madrigal *et al.* 2015). *Bacillus cereus s. l.* B25 produces two chitinases, ChiA and ChiB (Douriet-Gámez 2018), and the expression of the two chitinase-coding genes *chiA* and *chiB* is induced in response to the presence of chitin (Figueroa-López *et al.* 2017), which could explain its action against *Fv*. Regarding the control of pathogenic fungi, purified chitinases from fungi and bacteria have been shown to inhibit phytopathogenic fungal growth (Neeraja *et al.* 2010; Frederiksen *et al.* 2013).

Purified chitinases from insects, fungi and bacteria are currently used in various applications including the treatment of chitin-contaminated waste, the preparation of chitooligomers for the food and pharmaceutical industries (Prasad and Palanivelu 2015; Esawy *et al.* 2016; Mohammadzadeh, Agheshlouie and Mahdavinia 2017), malaria control and even applications as anticancer and immunomodulatory agents (Stoykov, Pavlov and Krastanov 2015; Tamadoni Jahromi and Barzkar 2018). Likewise, several reports have used nanoparticles loaded with chitinases for various purposes including the determination of total fungal load of a sample based on chitin digestion (Preety and Hooda 2018), and also as an agent against phytopathogenic fungi (Rostami *et al.* 2017; Manikanta *et al.* 2018). To date, the source of the chitinase (plant, fungi and bacteria) has not been shown to represent an advantage; however, the purification process has been shown to make a difference in cost and quality (Stoykov, Pavlov and Krastanov 2015). Nevertheless, whatever the biotechnological application, large-scale protein purification imposes severe and artificial physicochemical conditions in order to achieve the maximum yield. Thus, it is imperative to fully describe the biochemical characteristics and to determine the optimal pH and temperature conditions of target enzymes (Neeraja *et al.* 2010; Tamadoni Jahromi and Barzkar 2018).

In this work, we aimed to biochemically characterize the two chitinases produced by *B. cereus s. l.* strain B25 that could potentially be used to control the phytopathogenic fungus *Fv* infections in maize, as well as to establish their direct effect on fungal growth and development.

MATERIALS AND METHODS

Microorganisms and culture conditions

The *B. cereus s. l.* B25 strain is a maize rhizospheric bacterium that was previously isolated and selected from a bacterial collection as described in Figueroa-López *et al.* (2016), as an antagonist of *F. verticillioides*, is kept cryopreserved at −80°C and routinely cultivated in LB medium at 30°C with orbital shaking at 225 rpm. The term *sensu lato* (in a broad sense) is being used since after phylogenomic analysis (Douriet-Gámez *et al.* 2018) placement of this strain as one of the species belonging to the *B. cereus* group is not possible. *F. verticillioides* strains P03, DA42 and F31 were obtained as described in Leyva-Madrigal *et al.* (2015) and are also kept cryopreserved and are routinely grown on PDA at 30°C in the dark. *Fv* P03 was selected to conduct the present work due to the fact that *B. cereus s. l.* B25 was selected as a specific strain against this highly virulent fungus. *Fv* DA42 and P03 belong to a group of isolates which cause high severity of root rot in maize (>77%) and they are members of the *Fv* genetic group 2, while isolate F31 presents low severity of root rot in maize (26%) and belongs to the genetic group 3 (Leyva-Madrigal *et al.* 2015). *Escherichia coli* OneShot®TOP10 (ThermoFisher Scientific, Waltham, MA) and BL21 StarTM (DE3) strains were grown on LB medium at 37°C with orbital shaking at 225 rpm. 150 μg/mL ampicillin was used for *E. coli* when carrying a plasmid.

Gene cloning of bacterial chitinases

Bacillus cereus sensu lato B25 genomic DNA was isolated with the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) and used as a template for subsequent PCR amplification. First, the *chiA* and *chiB* coding sequences were directionally cloned into a pENTR™/D-TOPO® vector (Invitrogen, Carlsbad, CA). Additionally, the reverse primers were modified so that the coding sequence of a six-histidine (6xHis) tag was added to the 3′ end before the stop codon of each gene. Briefly, the coding sequence of each gene was PCR amplified with Platinum Taq DNA polymerase (Thermo Fisher Scientific) with primers ChiBTP-F (5′-CACCATGAGGTCTCAAAAATTCACACTGCTATTACTA-3′) and ChiBHis-R (*CTAGTGATGGTGATGGTGATGG* TTTTCGC-TAATTACGGCATTTAAAAGTTC) for *chiB*, and ChiATP-F (5′-CACCATGTTAAACAAGTTCAAATTTTTTTGTTGTAT TTTA-3′) and ChiAHis-R (5′-*TTAGTGATGGTGATG*TTTTTGCAAGGAAAGACCAT CAA-3′) for *chiA*. The TOPO adapter sequence for directional cloning is underlined and the sequence of the 6xHis tag appears in italics. The respective *chiA* and *chiB* PCR products were purified and cloned into pGEM®-T Easy vector (PROMEGA, San Luis Obispo, CA) to generate the constructions pGAFI-A (*chiA*) and pGAFI-B (*chiB*), and were transformed into OneShot®TOP10 chemically competent *E. coli* cells (Thermo Fisher Scientific). Plasmid constructions were verified by enzyme restriction patterns. Using the pGAFI-A and pGAFI-B plasmids as templates, *chiA* and *chiB* were amplified with AccuprimeTM Pfx Polymerase (Thermo Fisher Scientific) to obtain blunt-end PCR products, which were cloned into pENTR™/D-TOPO® to generate the entry clones pENAFI-A (*chiA*) and pENAFI-B (*chiB*). Then, the recombinant vectors were individually transformed into OneShot®TOP10 chemically competent *E. coli* cells. Plasmids from positive colonies were verified by sequencing and restriction. Finally, the entry clones pENAFI-A and pENAFI-B were used to carry out an LR recombination reaction (Thermo Fisher Scientific) to recombine the *chiA* and *chiB* genes separately into the Gateway®-adapted destination vector pDEST17 (which is an N-terminal 6xHis fusion vector), resulting in clones pDAFI-A (*chiA*) and pDAFI-B (*chiB*). This strategy allowed us to obtain ChiA and ChiB proteins tagged with a 6xHis tag at both of their C- and N-termini. Plasmids pDAFI-A and pDAFI-B were ultimately transformed into *E. coli* BL21 Star (DE3) chemically competent cells for subsequent protein purification.

As a control, we modified pDEST17 vector by removing the *ccdB* gene. CcdB is the toxin in the bacterial toxin-antitoxin system CcdA/B, which ensures that the pDEST17 vector will not replicate when empty. *CcdB* coding region was removed by a sequential digestion using *SmaI* and *SalI*. The cohesive 5′-end left by *SalI* was filled in with Klenow fragment. Then, the blunt-ended linearized plasmid was self-ligated. This vector without the *ccdB* gene was named pDEST17.5 and was transformed into *E. coli* BL21 Star (DE3) chemically competent cells.

Expression and purification of recombinant bacterial chitinases

The expression of the *B. cereus s. l.* B25 recombinant chitinases from *E. coli* BL21 StarTM (DE3) carrying plasmids pDAFI-A or pDAFI-B was carried out according to the manufacturer's instructions (*E. coli* Expression System with Gateway® Technology user guide, Invitrogen).

Recombinant *B. cereus s. l.* B25 ChiA and ChiB tagged with a 6xHis tag—from now on referred to as ChiA6xHis and ChiB6xHis—were purified from whole cell lysates of *E. coli* BL21 Star (DE3) according to the Ni-NTA Spin kit manufacturer's instructions (QIAGEN, Cat. 31 314, Hilden, Germany) under native conditions, with the only modification being made to the lysis buffer by lowering imidazole from 10 to 1 mM. All of the collected fractions were analyzed by SDS-PAGE at 12%. The purified enzymes were stored at 4°C until characterization. Protein concentration was quantified using the Bradford assay (Bio-Rad, Hercules, CA). Since both recombinant proteins were co-purified with other unknown proteins, and in order to avoid future misinterpretations, we performed an expression and purification control of *E. coli* BL21 Star (DE3) carrying the control vector pDEST17.5.

Western blotting

Previous analysis of the ChiA and ChiB sequences revealed the presence of a predicted signal peptide at the N-terminus. Therefore, we cloned both genes so that recombinant proteins were generated with a 6xHis tag at both the N- and C-termini. This procedure ensured that if these proteins were indeed secreted, i.e. the signal peptide was processed and the N-terminal 6xHis tag was lost, the protein would still have the histidine-tag at the C-terminus. The presence of the 6xHis tag on the recombinant chitinases was confirmed by western blot. The protein concentration of second elution fractions from each protein purification was quantified, and 7 μg of both ChiA and pDEST17.5 and 3.5 μg of ChiB were separated by SDS-PAGE. The gel was transferred onto a 0.2-μm nitrocellulose membrane using the iBlot2 Dry Blotting System (ThermoFisher Scientific), and the membrane was incubated in a 5% blocking solution (Difco Skim Milk, BD, Franklin Lakes, NJ). The 6xHis tag was detected using a mouse monoclonal anti-6xHis tag primary antibody (Abcam, Cat. Ab18184, Cambridge, UK) in a 1:10 000 dilution in 5% blocking solution. The secondary antibody was a goat anti-mouse IgG-HRP conjugated antibody (Abcam, Cat. Ab6789) in a 1:2000 dilution in 1X PBS. Blotted proteins were detected using the SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific). Bands were revealed by using a ChemiDoc XRS (Bio-Rad).

Chitinase activity assay

Chitinase activity was determined using a fluorometric chitinase assay kit (Sigma Aldrich, Cat. No. CS1030; St. Louis, MO) based on the enzymatic hydrolysis of the chitin substrates bound to 4-methylumbelliferone (4 MU). All of the enzymatic assays described hereafter were performed according to the manufacturer's instructions at 37°C in 100 mM citrate buffer (pH 5) for 30 min with a substrate concentration of 0.2 mg/mL, unless indicated otherwise. One unit of chitinase activity was defined as the amount of enzyme releasing 1 μmol of 4 MU from the substrate per minute. Each activity assay in this and the following enzymatic activity/kinetic parameter methods sections was performed at least three times in three independent experiments.

Substrate specificity assay

To determine the substrate specificity of ChiA and ChiB, chitinase activity was assayed using the three different substrates provided by the chitinase assay kit described above: N-acetyl-β-D-glucosaminide (GlcNAc), N,N′-diacetyl-β-D-chitobioside (GlcNAc-2) and β-D-N,N′,N″-triacetylchitotriose (GlcNAc-3). Briefly, each chitinase was incubated separately with 0.2 mg/mL of each substrate and incubated at 37°C in 100 mM

citrate buffer (pH 5) for 30 min. The reaction was stopped by adding the Stop solution, and the fluorescence was measured at an excitation and emission wavelengths of 360 nm and 450 nm, respectively.

Determination of kinetic parameters

The kinetic parameters of recombinant ChiA and ChiB for their specific preferred substrate(s) were determined as follows: each chitinase was incubated at 37°C in 100 mM citrate buffer (pH 5) for 30 min with its previously identified preferred substrate using the following substrate concentrations: for ChiA: 0, 1, 5 10, 20, 40 and 50 μM of GlcNAc-2; and for ChiB: 0, 1, 5 10, 20, 30 and 40 μM of both GlcNAc-2 and GlcNAc-3. The K_m and V_{max} values were calculated from kinetic data using the GraphPad Prism 7 software (https://www.graphpad.com/scientific-sofware/prism/).

Determination of the optimum pH and temperature for chitinase activity

The chitinase enzyme activity assay was performed in a pH range from 1.0 to 8.0 using hydrochloric acid-potassium chloride (0.1 M, pH 1.0–2.0) and citrate-phosphate (0.1 M, pH 3.0–8.0) buffers, in order to determine the optimum pH. Chitinase activity was assessed in a temperature range from 10 to 80°C in order to determine the optimal temperature.

Assay of antifungal activity: effect on conidia germination

We next performed a conidia germination inhibition assay as reported in Sousa *et al.* (2019) with slight modifications, to determine the antifungal properties of the recombinant ChiA and ChiB against the pathogenic fungus *F. verticillioides* strains P03, DA42 and F31. Briefly, 1000 conidia were placed in a microcentrifuge tube containing 50 μL of 2X Potato Dextrose Broth (PDB) prepared with 100 mM citrate-phosphate buffer at a final volume of 100 μL (final pH 5.0), adjusted with elution buffer from the protein purification. A total of four treatments were conducted in duplicate: (i) a control with no enzyme; (ii) 2 units of ChiA; (iii) 2 units of ChiB; (iv) 2 units total of both ChiA and ChiB (1 unit each). All tubes were incubated at 30°C for 48 h in the dark. After the incubation, conidia were visualized with a microscope with a Neubauer chamber and germination of 100 conidia was quantitated per tube. The percentage of germination was assessed for each sample with the following formula:

$$\%\ \text{of germination} = \frac{\text{germinated conidia}}{\text{observed conidia}} \times 100$$

This experiment was performed with three independent replicates (independent tubes) and the mean of the percentage of germination is reported. The experiment was repeated twice independently with similar results. The results were analyzed by a one-way ANOVA and a Tukey test ($P < 0.05$), using the SAS v9.0 software.

RESULTS

Expression and purification of recombinant chitinases

As shown in Fig. 1A, both recombinant chitinases were recovered in the elution buffer. Wild type ChiA and ChiB have a predicted molecular weight (MW) of 39.4 kDa and 74.2 kDa, respectively. In the SDS-PAGE, ChiA6xHis appears to have a MW near 49 kDa, whereas ChiB6xHis appears to be around 80 kDa.

Figure 1B displays the result of a western blot experiment using the anti-6xHis antibody to detect the purified recombinant proteins. Both ChiA6xHis and ChiB6xHis were successfully detected, whereas no bands were observed in the control lane. The western blot experiment thus allowed us to differentiate the specific band for each of our proteins from the various bands observed in the SDS-PAGE.

Table 1. K_m and V_{max} values for the two *B. cereus s. l.* B25 recombinant chitinases with their preferred substrate. GlcNAc-2, N,N′-diacetyl-β-D-chitobioside; GlcNAc-3, β-D-N,N′,N″-triacetylchitotriose.

Enzyme	Substrate	K_m (μM)	V_{max} (μmol/min/mg)
ChiA6xHis	GlcNAc-2	1.07	5.2
ChiB6xHis	GlcNAc-2	82.54	37
	GlcNAc-3	50.58	1.5

Substrate specificity of recombinant chitinases

As shown in Fig. 2, ChiA6xHis only showed enzymatic activity with GlcNAc-2, which is a suitable substrate for exochitinase activity (specifically chitobiosidase), whereas ChiB6xHis was active with both GlcNAc-2 and GlcNAc-3 as substrates, indicating that it is active as both an exo- (chitobiosidase) and endochitinase (Chernin *et al.* 1998).

Characterization of the purified recombinant chitinases

The K_m and V_{max} values for each of the two recombinant chitinases were determined by measuring their enzyme activity using the previously determined preferred substrates at different concentrations, varying from 1 to 50 μM for ChiA6xHis with GlcNAc-2; and 1–40 μM for Chib6xHis with both GlcNAc-2 and GlcNAc-3. The results are summarized in Table 1. ChiA6XHis showed higher affinity to the substrate GlcNAc-2 than ChiB6XHis (K_m values 1.07 μM vs 82.54 μM), although ChiB6XHis exhibited a higher V_{max} for GlcNAc-2 than ChiA6XHis. ChiB6XHis exhibited a K_m value for GlcNAc-3 of 50.58 μM and a V_{max} of 1.5 μmol/min/mg.

Figure 3 shows the activity of the purified recombinant chitinases at various temperatures (top panels). For ChiA6xHis, the optimal temperature was 20°C, while it did show considerable activity (e.g. over 50%) in the range of 20–40°C. The activity of ChiB6xHis with GlcNAc-2 as its substrate was over 40% at temperatures above 30°C, with the optimal temperature at 50°C and an activity remaining near 100% at 60°C. ChiB6xHis with GlcNAc-3 as its substrate exhibited less than 40% activity between 30 and 60°C, an optimal (~100%) activity at 70°C and a final drop in activity to less than 40% at 80°C.

The chitinase activity of ChiA6xHis varied from 60 to 100% in a pH range of 1–3, and dropped under 20% at above pH 4, as shown in Fig. 3, bottom panels. ChiB6xHis with GlcNAc-2 displayed a 40–100% activity in a pH range from 1 to 4 and then decreased. With GlcNAc-3, it exhibited an activity of greater than 40% at pH 2, followed by a gradual increase until reaching 100% activity at pH 4 and then a decline to 60% activity at pH 5 with an abrupt drop above pH 6.

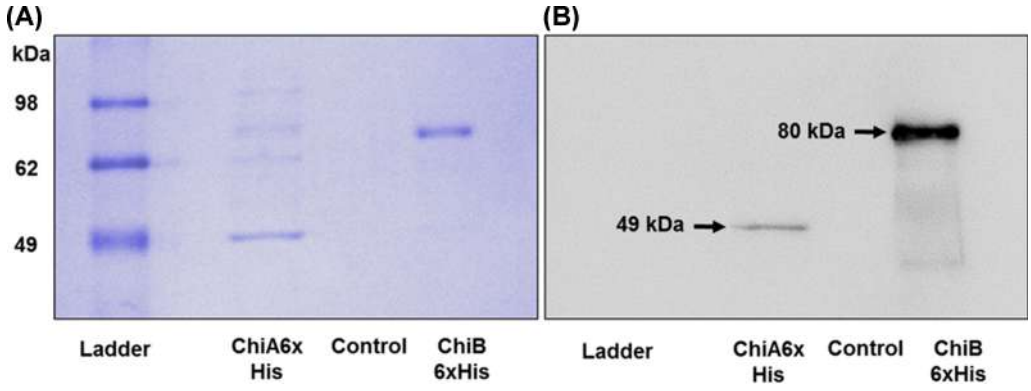

Figure 1. (A) SDS-PAGE showing the elution fraction from the purification of the recombinant chitinases ChiA and ChiB—tagged with a 6xHis tag—along with the purification of the control vector pDEST17.5. (B) Western blot to detect the 6xHis tag of the recombinant chitinases from the SDS-PAGE in (A). ChiA6xHis appears to have a MW near 49 kDa, whereas ChiB6xHis appears to be around 80 kDa. The two recombinant chitinases were detectable whereas no band was observed in the control.

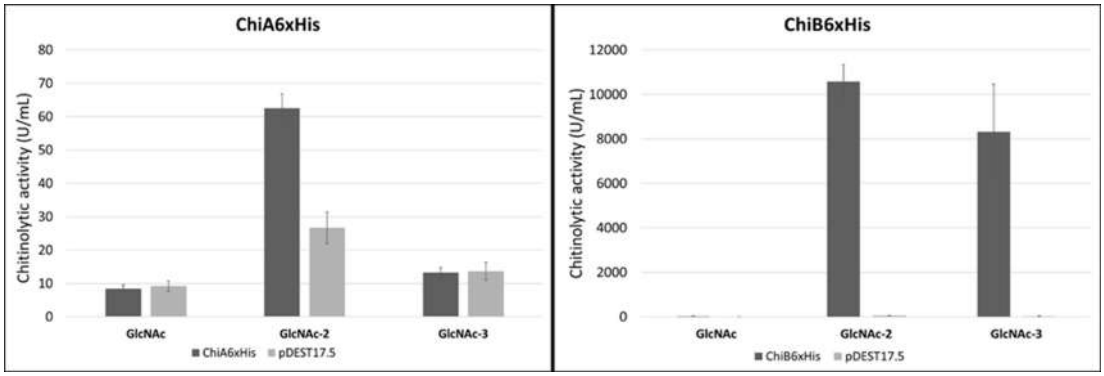

Figure 2. Determination of substrate specificity for the recombinant chitinases ChiA6xHis and ChiB6xHis using three different substrates: N-acetyl-β-D-glucosaminide (GlcNAc), N,N′-diacetyl-β-D-chitobioside (GlcNAc-2) and β-D-N,N′,N″-triacetylchitotriose (GlcNAc-3). The protein purification of the modified empty vector pDEST17.5 was used as control. Error bars represent standard deviation of three independent experiments.

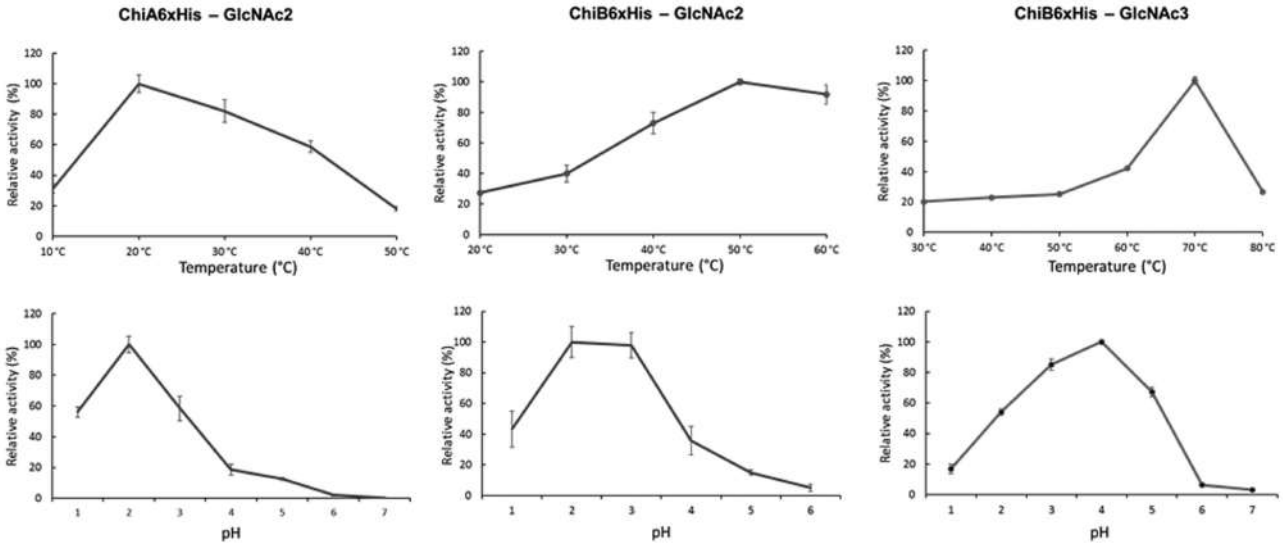

Figure 3. Effect of temperature (top) and pH (bottom) on chitinase activity. The chitinase enzyme activity assay was performed in a pH range from 1.0 to 8.0 and in a temperature range from 10 to 80°C. Error bars represent standard deviation of three independent experiments.

Table 2. Percentage of conidial germination of three different *Fusarium verticillioides* strains (P03, DA42 and F31) after incubation at 30°C for 48 h in darkness with the purified recombinant chitinases ChiA6xHis and/or ChiB6xHis. This experiment was performed with three independent replicates (tubes) counting 100 conidia per replicate using a Neubauer chamber and the mean of the percentage of germination with standard deviation is reported. The different superscript lowercase letters after standard deviation values indicate significant differences between treatments within each strain (Tukey's test, $P < 0.05$).

	Germination (%)		
Treatment	*Fv* P03	*Fv* DA42	*Fv* F31
No *enzyme*	58 ± 8.49[a]	33.50 ± 6.62[a]	38.83 ± 0.98[a]
ChiA6xHis	13 ± 1.40[c]	14.83 ± 0.98[c]	24.33 ± 3.93[b]
ChiB6xHis	33 ± 7.07[b]	21.67 ± 4.13[b]	22.17 ± 6.39[b,c]
ChiA6xHis + ChiB6xHis	12.5 ± 4.95[c]	15.67 ± 2.25[b,c]	17.17 ± 3.76[c]

Antifungal activity

We tested the ability of the purified chitinases to inhibit conidial germination in the phytopathogenic fungi *Fv* P03, DA42 and F31. As summarized in Table 2 we found that the treatments could minimize conidial germination of the three different strains of *Fv* (33.5–58%) to a range of 12.5–33%. For ChiA we obtained conidia germination inhibition percentages 38–78% depending on the isolate, whereas the percentage of inhibition for ChiB was between 35 and 43%. The combined ChiA and ChiB treatment showed conidia germination inhibition percentages from 53 to 79%. As shown in Fig. 4, both chitinases inhibited *Fv* P03 hyphal growth. In the control treatment, typical hyphae growth can be observed (top panel) alongside non-germinated conidia (bottom panel). In contrast, fewer germinated conidia were observed when *Fv* P03 was confronted with ChiA6xHis, ChiB6xHis, or the combined treatment. Moreover, the conidia that germinated in the presence of the chitinases were found to be swollen, unbranched and smaller than the control.

DISCUSSION

Bacterial chitinases are potential targets for biotechnological applications, from the biocontrol of phytopathogenic fungi (Neeraja *et al.* 2010; Banerjee and Mandal 2019) to disposal of chitin waste (Stoykov, Pavlov and Krastanov 2015).

The inhibition of fungal growth exerted by bacterial chitinases has been widely demonstrated (Senol *et al.* 2014; Rostami *et al.* 2017), and research on the kinetic properties of various bacterial chitinases is being conducted to fully exploit their characteristics for use in biotechnological applications (Rostami *et al.* 2017). In this work, we characterized the two chitinases produced by *B. cereus s. l.* B25, ChiA and ChiB, whose ability to antagonize the phytopathogenic fungus *Fv* P03 has previously been demonstrated (Figueroa-López *et al.* 2016).

First, both genes were cloned in *E. coli* to obtain two recombinant proteins with a 6xHis tag. We were able to express and purify both recombinant chitinases under native conditions, along with several other non-identified proteins that co-purified mostly with ChiA6xHis. Once purified, their substrate specificity was investigated and we found in Fig. 2 that ChiA was able to hydrolyze the substrate chitobioside (GlcNAc-2), indicating that it possesses exochitinase activity. ChiB was active against chitobioside and triacetylchitotriose (GlcNAc-2 and GlcNAc-3), as shown in Fig. 2, so it has been classified as a dual chitinase with exo- and endo-chitinase activities. In a previous work based on their sequences, ChiA was classified as an exochitinase and ChiB as an endochitinase (Figueroa-López *et al.* 2017). Different studies on the chitinases of *Serratia marcescens* (Horn *et al.* 2006) and *Streptomyces* sp. F–3 (Sun *et al.* 2019) have proposed that both exo- and endo-chitinase activities could work processively, i.e. that the enzyme does not release the substrate and proceed with the enzymatic reaction. In this perspective, ChiB could be acting as an endochitinase (cleaving triacetylchitotriose) that generates the substrate needed for its exochitinase activity (chitobiosidase). The previous sequence analysis of B25 ChiA and ChiB also showed that both chitinases belong to family 18 of the glycoside hydrolases, and that ChiB has two auxiliary functional domains: a carbohydrate binding domain (CBM_2), and a fibronectin type III domain (Fn3; Figueroa-López *et al.* 2017), which has been reported to enhance the binding of the substrate and the stability of the enzyme (Sun *et al.* 2019). These additional functional domains are proposed to be related to the interaction of the bacterial chitinase with the fungal chitin. In support of this, Fig. 2 shows that ChiB displayed a higher enzymatic activity than ChiA.

Regarding thermal stability, ChiA had an optimal temperature of 20°C, and its activity slowly decreased (down to 20%) as the temperature increased beyond 50°C. In contrast, ChiB, as an exochitinase, increased its activity from 30% at 20°C to 100% at 50°C, while as an endochitinase it had a basal activity of 30% at 20°C that increased to 100% at 70°C as shown in Fig. 3. The results for both chitinases are similar to those reported for chitinases from other *Bacillus* species, as well as *S. marcescens* (Bhattacharya, Nagpure and Gupta 2007; Hamid *et al.* 2013). In terms of pH, both ChiA and ChiB (as an exochitinase) performed better at acidic pH (2–3), although ChiB (as an endochitinase) displayed higher activity in a pH range from 2 to 5, as it was shown in Fig. 3. These results are different from those reported for other *Bacillus* (Wang *et al.* 2018) and *S. marcescens* (Emruzi *et al.* 2018) chitinases, which exhibited a nearly neutral optimal pH (6.5–7.5).

Altogether, these results suggest that ChiB, functioning as an endochitinase, randomly cleaves chitin chains at internal sites in the fungal wall, generating the substrate for the exochitinase activity of ChiB itself as well as for ChiA. It has also been reported that chitinases from the same family work synergistically to achieve maximum chitin degradation (Sun *et al.* 2019). Our results correlate well with the observations made by Figueroa-López *et al.* (2017), who showed that *chiB* transcripts accumulated 24 h after the addition of fungal lysate and colloidal chitin, whereas *chiA* transcript accumulation peaked at 72 h, suggesting a temporally concerted induction. Thus, it is possible that ChiB, as an endochitinase, is the main contributor to chitin degradation. Our K_m values show that ChiB has a higher affinity for its substrate as an endochitinase (GlcNAc-3) than as an exochitinase (GlcNAc-2). Additionally, ChiA has a lower affinity for GlcNAc-2 than ChiB as shown in Table 1. This supports our proposal that ChiB cleaves the chitin present in the fungal wall via its auxiliary functional domains CBM_2 and Fn3 (Yan and Fong 2015). ChiB randomly hydrolyzes internal glycoside bonds (endochitinase activity), forming chitobiose and low molecular mass multimers of GlcNAc that are the substrates for ChiA and ChiB itself (exochitinase activity).

We previously reported that *B. cereus s. l.* B25 has an antagonistic effect against *Fv* (Lizárraga-Sánchez *et al.* 2015; Figueroa-López *et al.* 2016), and we proposed that part of this phenomenon could be explained by the production of both chitinases. In this work, we show in Table 2 that both recombinant chitinases inhibit conidial germination of *Fv* strains P03, DA42 and

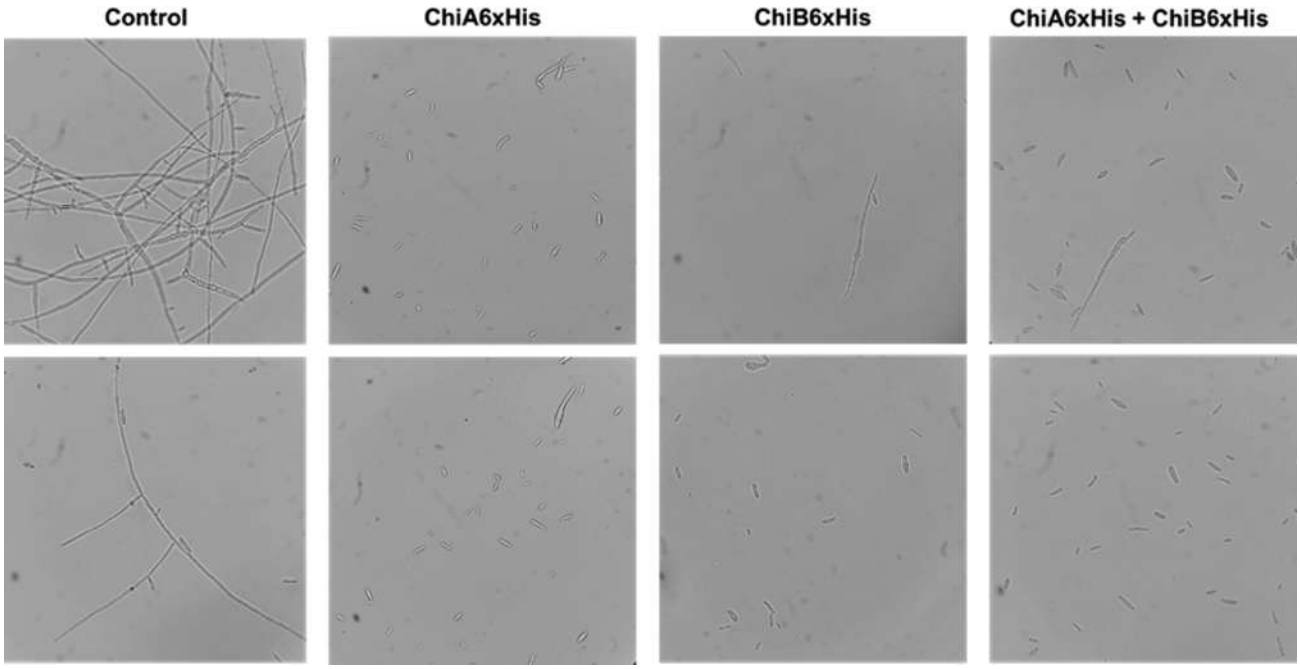

Figure 4. Microscopical observation of a conidia germination inhibition assay. *Fusarium verticillioides* P03 conidia were incubated at 30°C for 48 h with the recombinant chitinases ChiA6xHis and/or ChiB6xHis. The control consisted in a no enzyme treatment. A total of two different fields are presented for each treatment. The images are representative of two independent experiments.

F31. Indeed, we were unable to find fully germinated, branched hyphae of *Fv* as seen in the control when the conidia were incubated with ChiA6xHis, ChiB6xHis, or the combined treatment as shown in Fig. 4. Previous work has demonstrated up to 90% conidia germination inhibition in phytopathogenic fungi after incubation with purified bacterial chitinases (Zhang and Yuen 2000; Kishore, Pande and Podile 2005; Seo *et al.* 2016). Our results clearly show that both chitinases can degrade the cell wall and therefore inhibit fungal growth and conidia germination.

As a practical application, the construction and purification of bacterial chitinases allowed us to demonstrate *in vitro* their direct activity against *Fv* P03, and also allowed us to identify these chitinases that could potentially be used in industry and that could even be genetically modified to improve their activity for use as biological fungicides. Our results show that both *B. cereus s. l.* B25 chitinases play an important role in the antagonism against *Fv* P03, although further analysis of other extracellular compounds produced by this bacterium (Douriet-Gámez *et al.* 2018) is needed to fully understand this fungus-bacterium interaction.

Bacillus cereus s. l. B25 colonizes maize roots and forms biofilms at the root epidermis and apex, in addition to inhabiting the inside of the vascular tissue (Figueroa-López 2016), thus sharing the same ecological niche as *Fv* P03 in maize. *Fv* is able to colonize the root tissue, and once it reaches the vasculature it produces conidia and systemically invades the maize plant. Vascular tissue has an acidic pH of 4.5–6.0 (Marschner and Römheld 1983; Daeter, Slovik and Hartung 1993; Fan and Neumann 2004), suggesting that bacterial chitinase activity will be favored under these conditions, and that they may be more active at this site. This could help to explain the role of these bacterial chitinases inside the root tissue in fighting *Fv*, and the success of bacterial application in field experiments to control *Fusarium* stalk and ear rot in maize (Lizárraga-Sánchez *et al.* 2015). Along these lines, our research group is currently working on elucidating the mechanisms of how *B. cereus s. l.* B25 exerts its biological control over *Fv* in maize by studying the tripartite B25–*Fv* P03–maize interaction, in order to understand the role that bacterial chitinases may play in the control of the fungus when both microorganisms interact in the host plant.

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Conflicts of interest. None declared.

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