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Induction of *Bacillus cereus* chitinases as a response to lysates of *Fusarium verticillioides*

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Abstract

This study was carried out to evaluate the relative expression and enzymatic activity of Bacillus cereus sensu lato B25 chitinases in response to a Fusarium verticillioides (Fv) lysate. Phylogenetic studies revealed that B25 chitinases are closely related to chitinases from other Bacillus species clustering with type A and B chitinases from B. thuringiensis and B. cereus. The transcript levels of both ChiA and ChiB chitinases showed peaks of accumulation at 72 and 24 h, respectively, after the addition of either fungal lysate or colloidal chitin. Furthermore, an induction of exochitinase and endochitinase activity was detected in the supernatant of B25 after addition of colloidal chitin and fungal lysate and putatively attributed to ChiA and ChiB, respectively. These enzymatic activities were induced after 12 h and remained constant up to 72 h after the addition of treatments. Combined, the responses of ChiA and ChiB to Fv lysate suggest that these chitinases may partake in the antagonistic mechanism that B25 exerts upon Fv, possibly resulting in fungal growth inhibition.

Keywords:*Antagonism; stalk, ear and root rot (SERR) of maize; biocontrol; chitin; cell wall degradation*

1. Introduction

Maize has a central role in the Mexican agriculture, and its importance as a crop is reflected in the large land area dedicated to its cultivation. This crop is affected by multiple pathogens, of which one of the most common is *Fusarium verticillioides* (*Fv*), triggering the development of *Fusarium* stalk, ear and root rot (SERR) with significant economic losses (LIZÁRRAGA-SÁNCHEZ, et al. [1]).

Chitin is the main structural component of the fungal cell wall, a homopolymer of β-1,4 N-acetyl-D-glucosamine (GlcNAc), the second-most abundant polysaccharide in nature after cellulose (NAGPURE, et al. [2]). Fungi such as *Trichoderma* and bacteria from the genera *Bacillus*, *Serratia* and *Alteromonas* can hydrolyze chitin by means of chitinase production making them valuable for biotechnological control approaches, due to their potentially important role in the inhibition of pathogenic fungi (ASHWINI and SRIVIDYA [3]).

Chitinases from different *Bacillus* spp. exhibit antifungal activity against several phytopathogenic fungi (LIANG, et al. [4]). *Bacillus cereus* strain CH2 has been reported to suppress *Verticillium* spore growth (LI, et al. [5]), whereas chitinases from the strain YQ 308 inhibit the growth of phytopathogenic fungi such as *F. oxysporum*, *F. solani* and *Pythium ultimum*(CHANG, et al. [6]).

Recently, we screened a collection of 11,520 bacterial isolates from the rhizosphere of maize in order to identify a biological control agent for *Fusarium* SERR. From this work, three *Bacillus* isolates (*B. megaterium*, *Bacillus* sp. and *B. cereus sensu lato*) showing promising potential as antagonists against *Fv*, the causative agent of *Fusarium* SERR of maize were selected both *in vitro* and *in planta* (FIGUEROA-LÓPEZ, et al. [7]). Additional field trials have shown that strain B25 was the most effective bacterium at decreasing the incidence and severity of *Fusarium* stalk rot (FSR) and *Fusarium* ear rot (FER), as well as fumonisin levels in grain (LIZÁRRAGA-SÁNCHEZ, et al. [1]). Moreover, *in vitro* assays indicate that B25 produces siderophores and exhibits protease, glucanase and chitinase activities (FIGUEROA-LÓPEZ, et al. [7]). Based on these findings, coupled with the report that chitinase activity has an antifungal effect (NAGPURE, et al. [2]), we hypothesized that B25 chitinases are part of the antagonistic mechanisms used by this strain against *Fv*. Some properties and characteristic on *Bacillus* chitinases have been reported. Nevertheless, the relative expression has not been studied in *B. cereus* chitinases. This work investigates the effect of *Fv* lysates on B25 ChiA and ChiB transcript levels and their corresponding enzymatic activities.

2. Materials and Methods

2.1 Organisms and culture conditions: The bacterial strain was stored at −70 °C in Luria Bertani (LB) broth (Sigma, Cat. No. L3022, St. Louis, MO, USA) with glycerol (15 %, v/v), activated on LB agar and cultured overnight at 25 °C for 24 h. The following day, one single colony was taken and placed on LB broth to be cultured for 24 h at 30 ºC and a bacterial suspension containing 1 x 10^6 c.f.u./ml was used in the experiments.

Fungal isolate *Fv* P03 was previously molecularly identified (LEYVA-MADRIGAL, et al. [8]). This fungal strain is maintained as a frozen stock (-70 ºC) in potato dextrose broth (BD, Cat. No. 25492, Le Pont de Claix, France) containing 15% of glycerol.

2.2 Preparation of colloidal chitin and fungal lysate (chitin sources): Colloidal chitin (CC) was prepared from commercial chitin powder (Sigma Aldrich, Cat. No. C7170, St. Louis, MO, USA) according to WIWAT, et al. [9]. An *Fv* lysate was obtained from a 7-day old culture grown at 25 ºC in potato dextrose agar (BD, Cat. No. 213400, Le Pont de Claix, France). Mycelia were collected with a stainless steel spreader of bacteria in distilled water, dried in an oven at 50 ºC for 2 days, and subsequently ground up in a mortar (ANITHA and RABEETH [10]). Heat treatment does not damage chitin composition of the fungal cell wall (DEGUCHI, et al. [11]). Thus, in order to obtain sterile fungal lysate (FL) this was autoclaved at 121 ºC for 15 min and stored at 4 °C until use.

2.3 Chitinase induction assay:B25 chitinase induction assays consisted of the addition of two chitin sources as chitinase inducers: colloidal chitin (CC) and fungal lysate (FL); the B25 strain without inducers or chitin served as the control. The induction experiment was performed in 500 ml flasks at 30ºC for 72 h containing 100 ml of medium with the following components in g/l: 2 tryptone, 0.5 yeast extract, 1 NaCl, 0.125 KH_2PO_4, 0.125 K_2HPO_4, 0.05 calcium acetate and 0.05 magnesium acetate (SATO and ARAKI [12]). The B25 strain was grown for 8 h in the medium described above, after this the chitinase inducers (CC and FL) were added at a concentration of 0.1 % w/v. At different time points (0, 12, 24, 72 h), 1 ml

samples from each flask (three flasks per inducer used) were taken with a micropipette and placed in a 1.5 ml Eppendorf tube and centrifuged at 2,000 g for 5 min to separate bacterial cells, used for molecular analyses, from the culture supernatant employed for enzymatic activity assays. This experiment was performed by triplicate.

2.4 Quantitative PCR (qPCR): Cell pellets from 1 ml of cell culture were collected and 300 µl of lysis buffer (0.03 M Tris-HCl, 0.01 M EDTA and 20 g/l lysozyme) were added and incubated for 30 min at 37 ºC. Total RNA was isolated using TRIzol® Reagent (Thermo Fisher Scientific, Cat. No. 15596-026, Waltham, MA, USA), according to the manufacturer's instructions. RQ1 DNAse (PROMEGA, Cat. No. M6101, Fitchburg, WI, USA) was used to avoid DNA contamination. First-strand cDNA was prepared from total RNA using random hexamers with SuperScript™ III reverse transcriptase (Thermo Fisher Scientific, Cat. No. 18080-044, Waltham, MA, USA), following the manufacturer's instructions. Reagents and qPCR conditions were prepared as described in (CERVANTES-GÁMEZ, et al. [13]). All qPCR reactions were performed in a Rotor Gene-Q Real time PCR system instrument (Qiagen, Cat. No. 9001550, Hilden, Ger.) using SYBR Green Master Mix (Qiagen, Cat. No. 204074, Hilden Ger.). For PCR amplification, the thermocycler was programmed for 40 cycles at 95 °C for 5 s and 60 °C at 10 s, after an initial denaturation at 95 ºC for 5 min. Dissociation curves were performed at the end of each run to confirm single amplifications. The 30S ribosomal protein 21(rpsU) was used for data normalization (Table 1) (REITER, et al. [14]). Two primer pairs were designed for each gene, based on the B25 chitinase nucleotide sequences allowing for amplification of two different nucleotide regions (Table 1). The comparative threshold cycle method $2^{-\Delta\Delta Ct}$ was used to analyze relative mRNA expression, as previously reported (CERVANTES-GÁMEZ, et al. [13]). In this method, the expression of the chitinase gene was normalized according to rpsU gene expression across all treatment conditions. Subsequently, the normalized expression of each treatment was compared to that of the control condition. The result was used to determine the relative expression (i.e. the $2^{-\Delta\Delta Ct}$ value).

Table 1. Oligonucleotides used for qPCR

Gene	Position	Oligo sequence 5'→3'	Reference
Chi A	64f	CCTTTCCAAGCACAAGCAG	This study
	166r	TCCCATTTTGGTGAAACGTC	
Chi A	557f	GCATGGCTCCTGAAACAGC	This study
	692r	CTACCAGCGTTGTAGTGTTG	
Chi B	391f	TCAGGGACAACTTGGGAAG	This study
	513r	CCAAGTCCAGCCACCAAC	
Chi B	1561f	GCTGGAGAAGAGAAATGGAG	This study
	1673r	GATTTATTTCCAGCAGCATC	
rpsU		GTCTTTGGAGGATGCACTTCG	(REITER, et al. [14])
		GCTTTCTTGCCGCTTCAGAT	

2.5 Chitinase activity: The substrate-specific chitinase activity was determined using a chitinase assay kit (Sigma Aldrich, Cat. No. C7170, St. Louis, MO, USA). One unit of

chitinase activity was defined as the amount of enzyme required to release 1 μmol of 4-methylumbelliferone from the substrate per minute at pH 5.0 and 37 ºC. Each type of enzymatic activity was assayed using three biological replicates per sampling point in two independent experiments.

2.6 Phylogenetic analysis: Sequences were obtained from the B25 genome sequencing analysis conducted in our laboratory (unpublished results). Chitinase sequences were deposited in GenBank at the NCBI (National Center for Biotechnology Information) under accession numbers KR809875 (ChiA) and KR809876 (ChiB). Nucleotide sequences of the B25 chitinases were compared in GenBank using the BLAST-N and BLAST-X algorithms. MEGA 6.06 (TAMURA, et al. [15]) was used for alignment and phylogenetic analysis. Deduced amino acid sequences were aligned using the MUSCLE alignment program (EDGAR [16]). The phylogenetic tree was constructed using the Whelan and Goldman (WAG) model and the maximum likelihood (ML) method. Tree topology support was assessed by 1000 bootstrap replicates.

2.7 Statistical analysis: The results were analyzed using SAS software version 9 (SAS Institute Inc., Cary, NC, USA). Chitinase activity data were subjected to a repeated-measure analysis of variance (ANOVA, PROC MIXED procedure), to analyze the effects of treatment, time and their interaction on the measured variable. Data were fitted to different covariance structures and the best fit was used for further analysis. Heterogeneous autoregressive structure was assumed for endochitinase activity and Toeplitz with two bands structure for exochitinase activity. Tukey's adjusted least-square-means test was used to assess the differences between treatments ($P<0.05$). All tests were carried out using triplicate samples and were performed at least twice.

3. Results and discussion

3.1 Sequence analysis of B25 chitinases: The ChiA and ChiB chitinases from B25 share similar features with other chitinases reported from *B. cereus* CH (MABUCHI and ARAKI [17]) and *B. thuringiensis* serovar *sotto* (ZHONG, et al. [18]). The B25 ChiA gene contains a 1083 nucleotide-long open reading frame (ORF) that encodes a 360 amino acids peptide, with a calculated molecular mass of 39.4 kDa and a theoretical isoelectric point of 7.36 (Acc. No. KR809875). The ChiB gene contains a 2025 nucleotide-long ORF encoding a 674 amino acids peptide, with a calculated molecular mass of 74.2 kDa and a theoretical isoelectric point of 5.88 (Acc. No. KR809876). A putative Shine-Dalgarno sequence (AGGAG) located 8-9 bp upstream of the ATG initiation codon was previously predicted (HUANG, et al. [19]).

Our analysis of the ChiA and ChiB sequences revealed the presence of predicted signal peptides (SignalP 4.0) at their N-terminal regions (27 and 32 amino acids, respectively), providing evidence that these are secreted proteins. In addition, both ChiA and ChiB contain within their active sites three essential conserved amino acid residues in a DxDxE motif; this motif is highly conserved in a variety of chitinases (YAMABHAI, et al. [20]). The catalytic domain of ChiA shows homology with type A chitinases from *B. cereus* (MABUCHI and ARAKI [17]) and *B. thuringiensis* (MURAWSKA, et al. [21]).

Multiple sequence alignment revealed several amino acid substitutions that characterize the ChiB sequence: in position 13, the leucine observed in type B chitinases from other *Bacillus* strains is replaced by an isoleucine; and the Asp-190 within the ChiB active site differs from the other four *Bacillus* chitinases, which all contain Glu-190 (data not shown). The ChiB catalytic domain is categorized as belonging to the family of 18-glycosyl hydrolases on the basis of amino acid sequence (HENRISSAT and BAIROCH [22]). Similar

to other *Bacillus* chitinases, the ChiB protein contains a fibronectin type-III like domain (FnIII) and a cellulose-binding domain in the C-terminal region (data not shown) (DRISS, et al. [23]). These sequence and domain analyses confirm the categorization of B25 ChiA and ChiB as type A (exochitinase activity) and B (endochitinase activity) chitinases, respectively.

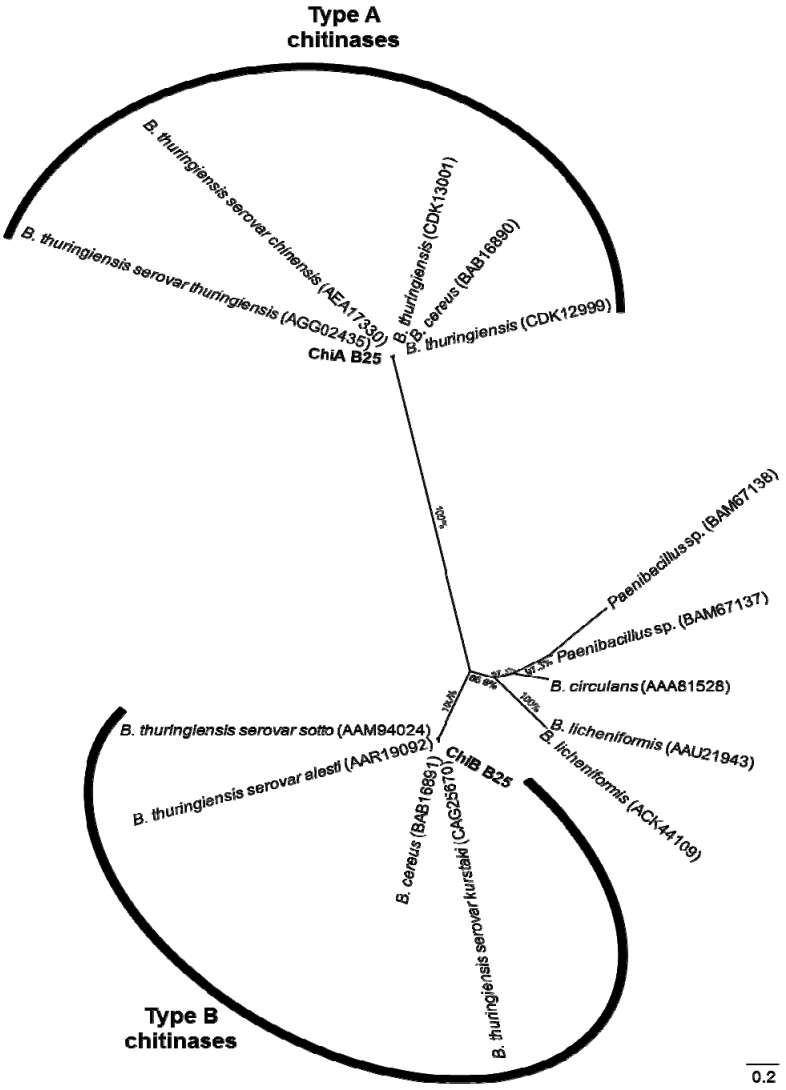

Figure 1. Maximum likelihood tree (log likelihood = -9212.82) based on complete amino acid sequences of type A and B chitinases from different *Bacillus* species. The tree was constructed with Mega 6.0 (bootstraps = 1000), using the Whelan and Goldman (WAG) substitution model with gamma distribution (+G). Chitinase sequences from *B. cereus* B25 are shown in boldface. The corresponding sequences of *Paenibacillus* sp. were used as an out-group. Database accession numbers of the sequences are provided in parentheses. Bootstrap values are shown as percentages. The scale bar indicates the expected number of amino acid substitutions per unit branch length.

3.2 Phylogenetic relationship of ChiA and ChiB: Phylogenetic analysis of the B25 ChiA and ChiB sequences indicates that they cluster with type A and type B chitinases, respectively, and are most closely related to chitinases from *B. thuringiensis* and *B. cereus* (Figure 1). These results complement our findings from the sequence and domain analyses of

ChiA and ChiB. Specifically, *B. thuringiensis* and *B. cereus* belong to the *B. cereus* group (also composed of *B. anthracis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis*), although it is difficult to differentiate the identity of these species. *Bacillus thuringiensis* produces crystal proteins during sporulation, and this feature is used to phenotypically distinguish it from *B. cereus* (RASKO, et al. [24]). Overall, several studies have revealed that these species are quite similar genetically, and may even constitute a single species (ZWICK, et al. [25]; LIU, et al. [26]), resulting in the term *B. cereus sensu lato* to describe members of this species complex.

3.3 ChiA and ChiB transcript levels increase in response to the inducers colloidal chitin (CC) and fungal lysate (FL): Chitinases play an important role in fungal pathogen control, and several studies have shown that application of fungal cell walls to bacteria induces bacterial chitinases (ANITHA and RABEETH [10]). The relative expression of the B25 ChiA and ChiB genes was evaluated by quantitative PCR, in order to investigate their responses when challenged with colloidal chitin and fungal lysate. Colloidal chitin was used as an induction control of chitinases transcription (LIU, et al. [27]). Both chitinases transcripts were detected from zero time, this supports their constitutive expression as reported before for other *B. cereus* strains (SATO and ARAKI [12]). In the presence of fungal lysate, ChiA transcript levels increased along time and a peak of induction at 72 h with 7.3-fold change, whereas in colloidal chitin the induction was 4.2-fold change relative to the rpsU control gene (Figure 2A). ChiB gene expression was induced by colloidal chitin and fungal lysate; an induction peak was found at 24 h, showing the highest induction when the fungal lysate was added with 8.6-fold change (Figure 2B).

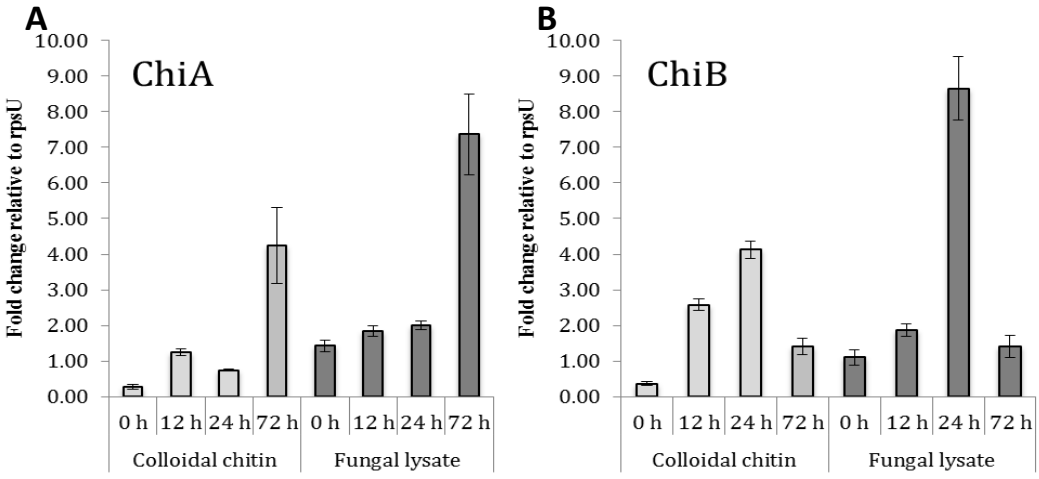

Figure 2. Differential expression of *B. cereus sensu lato* B25 ChiA and ChiB chitinases, induced by colloidal chitin and fungal lysate. The relative expression of ChiA and ChiB under these treatments (in comparison to rpsU in the control condition at each time) is presented in A) and B), respectively. Error bars indicate the standard deviation.

The sequential increase in ChiB (24 h) and ChiA (72 h) transcript levels suggests that both genes might act together to degrade chitin from the fungal lysate in a time-coordinated manner. It has been reported that gene expression in *B. cereus* CH chitinases is induced by a variety of chitin oligomers since 12 h of induction (SATO and ARAKI [12]). However, little information is available on how chitinase transcript levels change in response to phytopathogenic fungal lysates. Our results demonstrate that both colloidal chitin and fungal

lysate are good inducers of B25 ChiA and ChiB expression. Furthermore, these responses may be part of the mechanism that enables degradation of chitin in the fungal cell wall.

3.4 Extracellular chitinase activity: Next, we investigated the presence of extracellular chitinase activity in the supernatant culture media. We assumed that when the bacterium is grown in liquid medium added with colloidal chitin or fungal lysate, the supernatant of the culture media will contain ChiA and ChiB proteins, since both chitinases contain signal peptides that could allow for their extracellular allocation. We then quantitated the different types of chitinase activity using various fluorochromic substrates that can distinguish diverse endo- and exochitinase activities.A significant increase in both exo- (4-fold increase on average) and endochitinase (2-fold increase on average) activities was observed for both colloidal chitin and fungal lysate treatments with respect to the control condition (Table 2, significant treatment effect; Table 3). This increase was detected at 12 h and remained constant throughout time (Table 2, significant time effect; Table 3), as reported by SATO and ARAKI [12]. No significant differences were observed for the endo- and exochitinase activities after 12 h of induction at any other time between colloidal chitin and fungal lysate (Table 3).

Table 2. Summary of repeated measure analysis of variance (ANOVA) for chitinase activity of *Bacillus cereus sensu lato* B25 at four different times.

Effect	DF[a]	F[b]	P[c]
Endochitinase activity			
Treatment	2, 6	302.92	<0.0001
Time	3, 18	1215.04	<0.0001
Treatment *Time	6, 18	190.34	<0.0001
Exochitinase activity			
Treatment	2, 3	1164.75	<0.0001
Time	3, 9	1768.73	<0.0001
Treatment *Time	6, 9	131.49	<0.0001

[a] Numerator, denominator degrees of freedom (Proc Mixed, SAS).[b] Fisher test.[c] Probability.

On the other hand, we did not find a direct correlation between transcripts accumulation of ChiA and ChiB and chitinase activity. Exochitinase activity was detected at the starting point of the experiment when the colloidal chitin and fungal lysate were added. Other secreted chitinases from *B. cereus* and *B. thuringiensis* (WANG, et al. [28]) sharing high homology (98%) with ChiA from this report (Data not shown) also act as exochitinases (LI, et al. [5]). We only can suggest that ChiA may act as an exochitinase based on its similarity to other ChiA proteins. The peak of induction for the ChiB gene was observed at 24 h (Figure 2B). Using a combination of gene cloning and expression analysis, CHEN, et al. [29] demonstrated that the activity of a *Bacillus cereus* ChiB gene sharing a 97% amino acid sequence similarity with the ChiB gene from this study. Other chitinases similar to ChiB have been characterized

as endochitinases from *Bacillus cereus* and *B. thuringiensis* (CASADOS-VÁZQUEZ, et al. [30]). It is possible to suggest B25 ChiB may act as an endochitinase.

The lysis process of insoluble chitin consists of three main steps: (1) cleavage of the polymer into water-soluble oligomers; (2) splitting of these oligomers into dimers; and (3) cleavage of dimers into monomers (BEIER and BERTILSSON [31]). We suggest that ChiB could possibly act as an endochitinase that generates chitin dimers and/or oligomers; after their release, these products would then become substrates for exochitinases such as ChiA, which could degrade them into monosaccharides. This agrees with enzymatic activity measurements showing the induction of endo- and exochitinase activities after 12 h of culture under colloidal chitin and fungal lysate treatments (Table 3). Since transcripts for both chitinases are present from the beginning of the experiment (Figures 2A, B) it is possible to suggest that: 1) ChiA and ChiB transcripts level might be sufficient to cause an accumulation in the ChiA and ChiB protein amount and an increase in their enzymatic activity (Table 3); 2) the presence of the ChiA and ChiB proteins since the beginning of the experiment (Table 3) can cause the accumulation in time of chitin oligomers that may induce enzyme activity as reported by SATO and ARAKI [12]; 3) pre-made ChiA and ChiB proteins are only activated by the addition of colloidal chitin or fungal lysate (NIELSEN, et al. [32]).

Table 3. Chitinase activity measured in supernatants samples of induction experiment. Exochitinase activity using 4-Methylumbelliferyl N,N-´diacetyl-β-D-chitobioside as the substrate. Endochitinase activity using 4-Methylumbelliferyl β-D-N,N´, N´´-triacetylchitotriose as the substrate.

	Chitinase activity (U/ml)							
Treatment	Endochitinase				Exochitinase			
	0 h	12 h	24 h	72 h	0 h	12 h	24 h	72 h
Control	6.6±1.24 a, A	20.6 ±0.72 a, B	18.8±0.62 a, B	12.7±0.01 a, B	33.8±0.59 a, A	119±4.47 a, B	119.9±1.92 a, B	121.9±0.74 a, B
Colloidal chitin	4.5±0.30 ab, A	79.2±5.32 b, B	75.1±7.93 b, B	79.9±1.93 b, B	34.9±0.94 a, A	284±3.58 b, B	270.1±0.04 b, B	272.4±6.34 b, B
Fungal lysate	2.5±0.15 b, A	84.5±2.6 b, BD	72.8±0.58 b, C	80.8±10.1 b, CD	32.6±0.91 a, A	274.4±9.14 b, B	280.1±13.91 b, B	262.8±2.74 b, B

Different lower case letters in the same column indicate differences (P<0.05) between treatments at a given time.Different upper case letters in the same line indicate differences (P<0.05) between times in a given treatment. U: the amount of enzyme needed to release 1 µmol 4-methylumbelliferone from the substrate per minute at pH 5.0 and 37 °C.

The relative expression peaks for ChiB at 24 h and ChiA at 72 h could be related to the increase on chitin oligosaccharides generated by ChiB subsequently used by ChiA: however we cannot currently substantiate this since we found no direct correlation between the relative expression of chitinase and enzymatic activity. Recent advances in post-translational studies of the regulatory processing in mRNA and proteins, found that the abundance of protein may or not correlate with the mRNA levels due the RNA is less stable than proteins (VOGEL and MARCOTTE [33]). The mRNA half-life of ChiA and ChiB in *B. cereus* have not been studied but in *B. subtilis* is about 7 min (HAMBRAEUS, et al. [34]), while in *B. licheniformis* the half-life of the chitinase protein has been calculated as long as 20 days when grown at 37 ºC (NGUYEN, et al. [35]).

4. Conclusions

We identified two chitinases in the genome of *Bacillus cereus sensu lato* B25, ChiA and ChiB, which were putatively identified as exo- and endochitinase respectively, by sequence analysis and comparison to other sequences previously reported for other *Bacillus* species. Both chitinases were induced by colloidal chitin and fungal lysate, showing the possible role of these enzymes on fungal inhibition as a part of a broad range of mechanisms that the bacterium employs to inhibit fungal growth. The lack of correlation between the expression and enzymatic activity results may be due to the different mechanisms of RNA and protein processing. To confirm these findings, cloning, expression, purification and enzymatic characterization of these two genes are currently being addressed in our laboratory.

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