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Metalloprotease production by *Paenibacillus larvae* during the infection of honeybee larvae

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American foulbrood is a bacterial disease of worldwide distribution that affects larvae of the honeybee *Apis mellifera.* The causative agent is the Gram-positive, spore-forming bacterium *Paenibacillus larvae*. Several authors have proposed that *P. larvae* secretes metalloproteases that are involved in the larval degradation that occurs after infection. The aim of the present work was to evaluate the production of a metalloprotease by *P. larvae* during larval infection. First, the complete gene encoding a metalloprotease was identified in the *P. larvae* genome and its distribution was evaluated by PCR in a collection of *P. larvae* isolates from different geographical regions. Then, the complete gene was amplified, cloned and overexpressed, and the recombinant metalloprotease was purified and used to generate anti-metalloprotease antibodies. Metalloprotease production was evaluated by immunofluorescence and fluorescence *in situ* hybridization. The gene encoding a *P. larvae* metalloprotease was widely distributed in isolates from different geographical origins in Uruguay and Argentina. Metalloprotease was detected inside *P. larvae* vegetative cells, on the surface of *P. larvae* spores and secreted to the external growth medium. Its production was also confirmed *in vivo,* during the infection of honeybee larvae. This protein was able to hydrolyse milk proteins as described for *P. larvae,* suggesting that could be involved in larval degradation. This work contributes to the knowledge of the pathogenicity mechanisms of a bacterium of great economic significance and is one step in the characterization of potential *P. larvae* virulence factors.

INTRODUCTION

American foulbrood is one of the most severe bacterial diseases that affects honeybee larvae. It has a worldwide distribution, causing significant losses to honeybee populations and the beekeeping industry (Hansen & Brødsgaard, 1999). The causative agent is *Paenibacillus larvae*, a Gram-positive, spore-forming bacterium (Genersch *et al.*, 2006).

Larvae become infected when they consume food contaminated with *P. larvae* spores. These spores germinate in the larval midgut; vegetative cells proliferate, move to the haemocoel and spread throughout the larval body (Yue *et al.*, 2008). During proliferation, *P. larvae* secretes different proteins that may act as virulence factors (Antúnez *et al.*, 2010, 2011). It has been proposed that among the mixture of secreted proteins, *P. larvae* secretes metalloproteases that could be responsible for the larval degradation that occurs after death (Antúnez *et al.*, 2009; Dancer & Chantawannakul, 1997; Holst & Sturtevant, 1940; Hrabák & Martinek, 2007). Larvae with late-stage infections show a strong proteolytic activity that can be seen when larval debris is inoculated into milk, when protein coagulation occurs in a few hours (Holst & Sturtevant, 1940). Since metalloproteases seem to be related to the pathogenicity of *P. larvae*, the aim of the present work was to identify the sequence that encodes a metalloprotease in the *P. larvae* genome, and to evaluate its expression during the infection of honeybee larvae.

Abbreviations: FISH, fluorescence *in situ* hybridization; PFA, paraformaldehyde.

METHODS

Bacterial strains and plasmids. Fifty Uruguayan *P. larvae* isolates obtained from bees, larvae and honey of different geographical regions of Uruguay were selected from the collection of the Department of Microbiology, IIBCE (Antúnez *et al.*, 2004, 2007). Four Argentinian isolates corresponding to different genotypes (PL8, PL99 and PL63) (Alippi & Aguilar, 1998) and one additional isolate, PLP (formerly *P. larvae* subsp. *pulvifaciens*) were also used, in order to complete the four different *P. larvae* genotypes described (ERIC I - BOX A; ERIC I - BOX C; ERIC II - BOX B; ERIC III - BOX PLP: Antúnez *et al.*, 2007). *P. larvae* strain 44, isolated from honey (Durazno, Uruguay), was selected from further assays. *P. larvae* was routinely grown in J medium and incubated at 37 °C (Hornitzky & Nicholls, 1993).

Plasmid pET21(a)+ was used for DNA cloning and protein expression (Studier & Moffatt, 1986). *Escherichia coli* XL-1 Blue was used for initial cloning of the DNA into plasmid vectors and for maintenance of the plasmids (Bullock *et al.*, 1987). *E. coli* BL21(λD3)

was used for final transformation and overexpression of recombinant proteins. *E. coli* was grown in Luria–Bertani (LB) medium at 37 °C. LB was supplemented with ampicillin (Amp, 100 mg ml^{-1}) when necessary.

Preparation of *P. larvae* cells and protein suspensions. A *P. larvae* vegetative cell suspension in PBS was incubated at 4 °C for 30 days to get a spore suspension. To confirm the absence of vegetative cells and determine the spore number, an aliquot was fixed with 4 % paraformaldehyde (PFA) for 30 min, stained with DAPI (4′,6-diamidino-2-phenylindole, Sigma) (1 μg ml^{-1}) for 5 min and visualized by fluorescence microscopy.

A *P. larvae* liquid culture was incubated at 37 °C with shaking for 72 h. It was centrifuged, and the pellet was resuspended in PBS and sonicated to obtain the whole bacterial proteins.

Amplification of the metalloprotease gene. The distribution of a metalloprotease gene among the different *P. larvae* isolates was evaluated by PCR using primers Prot1 (5′-GCAGCAAATCGTATTCAG-3′) and Prot2 (5′-GGTCCTTTGTAACGATTG-3′), designed to amplify a 280 bp fragment of the unique nucleotide sequence published for this protein (GenBank accession number AF111421). Two different PCR products were selected and sequenced at Macrogen (Korea) in order to confirm their identity.

The partial nucleotide sequence encoding a *P. larvae* metalloprotease (GenBank accession number AF111421) was used to identify the complete gene that encodes this protein in the *P. larvae* genome (available at http://www.hgsc.bcm.tmc.edu/; Qin *et al.*, 2006). As the genome is not completely assembled, it was necessary to assemble two different contigs in order to get the complete sequence (Ctg01.584 and Ctg01.323). The sequence obtained was analysed using the Artemis program (available at www.sanger.ac.uk) in order to find ORFs. One ORF was identified as the metalloprotease gene (1590 bp).

Primers Met-F (5′-GGAGGCATATGAATGAAGAAG-3′) and Met-R (5′-CCTTAAGCTTA*GTGATGGTGATGGTGATG*TTTGACTCCAACGGCGTC-3′) were designed in order to amplify the complete gene, and included restriction sites for *Nde*I and *Hin*dIII, respectively (restriction sites are underlined). A histidine tag was included in primer Met-R to facilitate protein purification (shown in italics).

P. larvae DNA was extracted from pure cultures using the Genomic DNA extraction kit from Sigma. PCRs were carried out as previously described (D'Alessandro *et al.*, 2007; Piccini *et al.*, 2002), using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). Amplified DNA was analysed by electrophoresis on 0.8 % agarose gels, stained with ethidium bromide (0.5 μg ml^{-1}) and visualized using a UV-transilluminator (Sambrook *et al.*, 1989).

Cloning and overexpression of the metalloprotease gene and purification of the recombinant protein. PCR product was purified using the Gel Extraction DNA kit from Fermentas, digested with *Nde*I and *Hin*dIII (New England Biolabs) and cloned into plasmid pET21(a)+ (previously digested) using T4 DNA ligase (New England Biolabs). The ligation mixture was transformed into *E. coli* XL-1 Blue. Then the pET21(a)+ construct containing the *P. larvae* metalloprotease gene was moved into *E. coli* BL21(λD3) by transformation.

A single colony was inoculated into LB Amp and incubated with shaking until the OD_{600} reached 0.6. Protein production was induced by IPTG addition (final concn 1 mM). To corroborate the protein production, samples were taken before and at different times after that, centrifuged, and the pellets subjected to SDS-PAGE. To evaluate the protein solubility, the pellet taken 4 h after induction was resuspended in PBS, sonicated, centrifuged again, and pellet and supernatant were subjected to SDS-PAGE. The recombinant metalloprotease was purified under denaturing conditions and renatured in the Unidad de Producción de Proteínas Recombinantes, Institut Pasteur Montevideo, Montevideo, Uruguay. Briefly, the culture pellet was used to extract the inclusion bodies under denaturing conditions using 6 M guanidine.HCl. Inclusion bodies were suspended and renatured in 50 mM Tris, 500 mM NaCl, 20 % (v/v) glycerol, 0.7 M L-arginine pH 7.4, by the drop-by-drop method. The metalloprotease was dialysed in successive steps to eliminate the salts and concentrated (twice) with PEG 40 000 at 6 °C overnight. It was purified using an anionic interchange column with DEAE TSKgel-5PW resin (TOSOH Biosep), packed in a XK 16/100 column (GE Healthcare).

Protein concentration was determined by the method of Bradford (1976) using BSA as a standard, and the protein was stored at 4 °C.

Anti-metalloprotease serum preparation for immunofluorescence. Female CD-1 mice, 6–8 weeks old, from the breeding facilities at the IIBCE were used. Mouse antiserum was raised using a four-dose immunization schedule with 100 μg recombinant metalloprotease per dose. The first dose was administered in Freund's complete adjuvant, and subsequent doses, at 2-weekly intervals, in Freund's incomplete adjuvant. The serum was collected 1 week after the last immunization. Animal experiments were conducted in accordance with procedures authorized by the IIBCE.

Antiserum specificity evaluation. Antiserum specificity was evaluated by SDS-PAGE and Western blotting. Whole *P. larvae* proteins were suspended in sample buffer, boiled for 5 min, subjected to 12 % SDS-PAGE and stained with 1 % Coomassie brilliant blue (Sambrook *et al.*, 1989). Proteins subjected to SDS-PAGE were transferred to nitrocellulose membranes (Towbin *et al.*, 1979) and Western immunoblots were performed using a 1 : 100 dilution in PBS/Tween 20/1 % skimmed milk of mouse polyclonal antiserum raised against the recombinant metalloprotease.

Serum specificity was also tested using *P. larvae*-infected and non-infected larvae. Three *P. larvae*-infected larvae (collected in the field and with clinical symptoms of American foulbrood) and three non-infected ones were separately macerated in 500 μl PBS; an aliquot was mixture with sample buffer, boiled for 5 min and subjected to 12 % SDS-PAGE (Sambrook *et al.*, 1989). Proteins were stained with Coomassie brilliant blue and subjected to SDS-PAGE and Western blots using anti-metalloprotease serum, as described above.

Detection of metalloprotease by immunofluorescence. *P. larvae* vegetative cells or spore suspensions were fixed with 4 % PFA for 20 min. *P. larvae* cells or sections of larvae were washed with PBS, blocked with 2 % BSA in 50 mM PBS/NH_4Cl for 20 min and incubated with the primary antibody, the anti-metalloprotease serum (at 1 : 100 dilution), for 60 min in 50 mM PBS/NH_4Cl with 2 % BSA. After PBS rinses, cells were incubated with a FITC-conjugated secondary antibody to mouse IgG (1 : 100 dilution; Molecular Probes) for 30 min and finally rinsed with PBS. Incubations were performed at room temperature. A negative control was performed by treating the cells only with the secondary antibody. Imaging analysis was performed using a confocal microscopy (Olympus Fluoview 300).

Larval infection assay. Worker larvae from the progeny of a single wild-mated honeybee queen (*Apis mellifera ligustica*) maintained in a disease-free apiary at the IIBCE were used for this study. Larvae were collected and maintained as described by Evans (2004). Two groups of 12 larvae each were used. One larval group was fed with food supplemented with *P. larvae* at a final concentration of 1000 spores μl^{-1} for the first 48 h; thereafter, normal larval diet was used for feeding. The second group, control larvae, was fed with normal larval diet throughout the experiment. Each day, the larvae were taken out of the incubator and examined. Surviving larvae were transferred to

new wells filled with fresh food. The experiment was performed in triplicate. Infected and control (non-infected) larvae were sacrificed 5 days after infection, fixed by microinjection with 4 % PFA and incubated overnight in 4 % PFA. The larvae were extensively washed with PBS and embedded in a gelatin/albumin matrix. The matrix was prepared as follows. A mixture of 1 ml 4.5 % albumin in saline buffer (0.15 M NaCl, 0.003 M NaN_3), 0.5 ml 0.5 % gelatin in saline buffer and 50 µl 25 % glutaraldehyde was placed in a mould (1 × 1 cm) and larvae (previously dried with tissue paper) were placed in the middle of the mould. The mixture was air-dried for 5 min and then the matrix was removed from the mould. Histological sections (50 µm) were prepared using a vibratome and sections were mounted on glass slides previously covered with 2 % agarose.

FISH and immunofluorescence assays on larval sections. For visualization of bee midgut cells, the eukaryotic probe EUK516 5′-labelled with Cy3 (indocarbocyanine) was used. Larval sections were incubated with 100 ng of the probe in hybridization buffer (20 % deionized formamide, 0.9 M NaCl, 20 mM Tris/HCl pH 7.9 and 0.01 % SDS) at 46 °C in a humid chamber for 4 h. The sections were then washed with washing buffer and incubated at 48 °C in a humid chamber for 30 min. Finally, the sections were washed with distilled water and subjected to immunofluorescence analysis as described above.

Evaluation of proteolytic activity. The milk hydrolysis assay was carried out by a modification of the method described by Castro-Sowinski & Cantera (1995). Skim milk was prepared from powder dissolved in distilled water at a final concentration of 10 %; 1 ml prepared skim milk was added to 500 µl PBS and inoculated with 100 µl recombinant metalloprotease in PBS. A negative control was carried out using PBS instead of the recombinant protein. All assays were incubated at 37 °C for 24 h. The tubes were then centrifuged at 10 000 ***g*** for 5 min. The assays were considered as positive when milk proteins precipitated.

RESULTS

Distribution of a *P. larvae* metalloprotease gene

According to the PCR results, all Uruguayan and Argentinian *P. larvae* isolates tested carried the gene encoding the studied metalloprotease, indicating that this potential virulence factor is widely distributed (data not shown). Two PCR products were randomly selected and sequenced; they showed 99 % identity with the published partial sequence for *P. larvae* metalloprotease (AF111421.1).

Amplification, cloning and overexpression of a metalloprotease gene and purification of the recombinant protein

The complete gene encoding a *P. larvae* metalloprotease was amplified, cloned into pET21(a)+ and transformed into *E. coli* BL21(λD3). The production of the recombinant protein was confirmed by SDS-PAGE, since the expected 59 kDa band was observed after protein expression induction. The highest production was obtained after 4 h incubation (Fig. 1a). Most of the recombinant metalloprotease was detected in the insoluble protein fraction (data not shown) and it was successfully purified (Fig. 1b). After several days at 4 °C, the 59 kDa purified protein suffered autoproteolysis, yielding a 40 kDa protein (Fig. 1c).

Detection of the metalloprotease in *P. larvae* vegetative cells and spores by immunofluorescence

Mouse polyclonal anti-metalloprotease serum was used to determine the localization of the metalloprotease in *P. larvae* vegetative cells and spores. The protein was detected inside vegetative cells and on the surface of spores, as shown by immunofluorescence images (Fig. 2). The initial vegetative cell and spore suspensions were washed several times in order to clean the cells, since when no washes were carried out the green signal corresponding to secreted metalloprotease was detected throughout the surrounding medium, indicating the secretion of metalloprotease (data not shown). *P. larvae* cells treated only with the secondary antibody did not show any fluorescent signal (data not shown).

In order to test the specificity of the antiserum against *P. larvae* metalloprotease, SDS-PAGE and Western blot assays were performed. Among the mixture of whole *P. larvae* proteins only one protein was recognized (Fig. 3). In order to confirm that the antiserum did not recognize larval proteins, total larval proteins were obtained from *P. larvae*-infected and

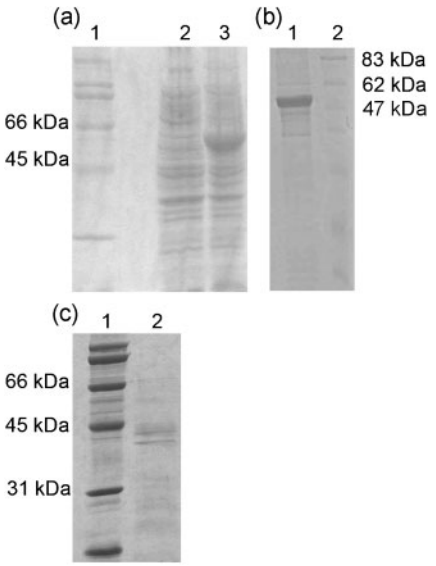

Fig. 1. Overexpression of *P. larvae* metalloprotease and purification of the recombinant protein. The *P. larvae* metalloprotease gene was amplified, and cloned into the pET21(a)+ system; expression was induced by IPGT addition and then evaluated by SDS-PAGE. Purification of recombinant protein was performed under denaturing conditions, and recombinant protein was incubated at 4 °C. (a) Overexpression of metalloprotease gene. Lanes: 1, Wide Range protein molecular mass markers (Bio-Rad); 2, before induction; 3, 4 h after induction. (b) Purification of recombinant metalloprotease. Lanes: 1, purified protein; 2, pre-stained protein markers, Broad Range (New England Bioloabs). (c) Recombinant metalloprotease after incubation at 4 °C for several days. Lanes: 1, Wide Range protein molecular mass markers (Bio-Rad); 2, purified protein.

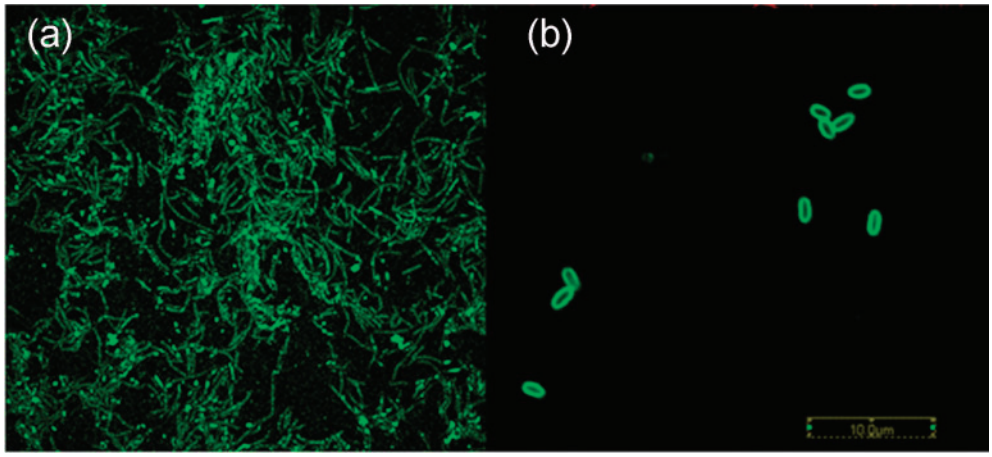

Fig. 2. Detection of metalloprotease in *P. larvae* vegetative cells (a) and spores (b) by immunofluorescence (original images taken at ×1000 and ×2000 magnification, respectively; scale bar in panel b represents 10 μm). *P. larvae* vegetative cells and spores were incubated with mouse polyclonal antiserum raised against *P. larvae* metalloprotease and FITC-conjugated secondary antibody against mouse IgG, and visualized by confocal microscopy.

non-infected larvae and subjected to SDS-PAGE and Western blotting using anti-metalloprotease serum. When the mixture of total proteins of *P. larvae* infected larvae was analysed, only one protein was detected, while no band was detected when non-infected larvae were analysed (Fig. 4). These results confirm the expression of metalloprotease by *P. larvae*, and also confirm the specificity of the antiserum.

Detection of the metalloprotease during infection of larvae by immunofluorescence and FISH

To evaluate the production of *P. larvae* metalloprotease during infection of honeybee larvae, the infection was reproduced in the laboratory and 5-day-old larvae were analysed by inmmunofluorescence and FISH. Metalloprotease was detected inside the larval midgut, as revealed by confocal microscopy images, confirming the production of this protein *in vivo* (Fig. 5). No metalloprotease production was detected in the midgut of non-infected larvae.

Evaluation of the proteolytic activity of the metalloprotease

Recombinant metalloprotease was able to hydrolyse milk proteins, as shown by the precipitation of proteins, confirming its proteolytic activity (Fig. 6). A negative control using PBS or protein elution buffer instead of the metalloprotease did not cause coagulation of milk.

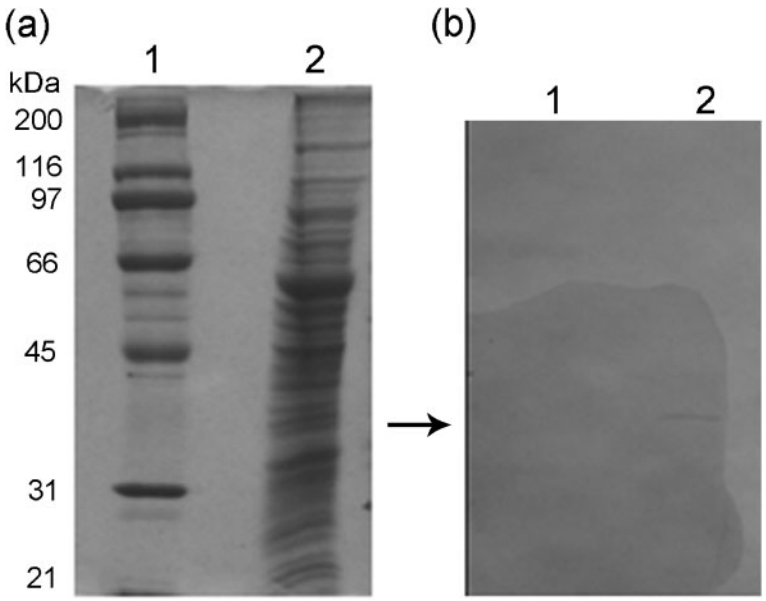

Fig. 3. Specificity of the antiserum used for *P. larvae* metalloprotease detection, using whole *P. larvae* proteins. The arrow indicates the recombinant metalloprotease. (a) SDS-PAGE analysis. (b) Western blot analysis. Lanes (both panels): 1, Wide Range protein molecular mass markers (Bio-Rad); 2, *P. larvae* whole bacterial proteins.

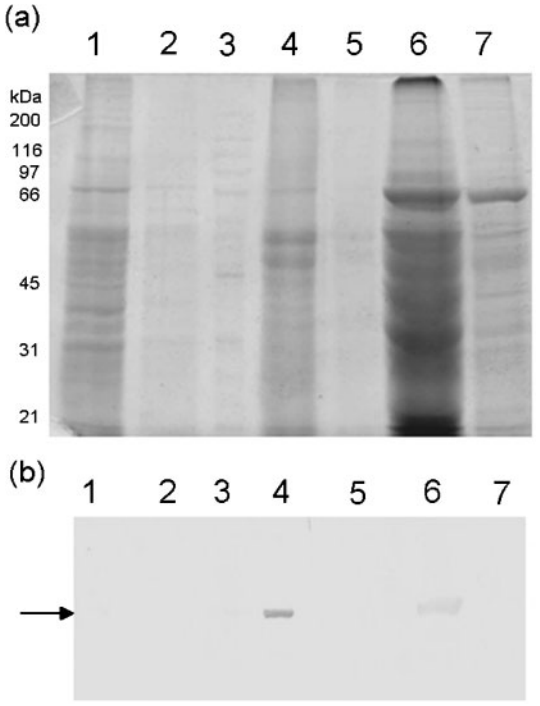

Fig. 4. Specificity of the antiserum used for *P. larvae* metalloprotease detection, using whole protein preparations from infected and non-infected larvae. The arrow indicates the protein band corresponding to recombinant metalloprotease. (a) SDS-PAGE analysis. (b) Western blot analysis. Lanes (both panels): 1, total proteins of non-infected larvae; 2, total proteins of non-infected larvae (1/10 dilution); 3, Wide Range protein molecular mass markers (Bio-Rad). 4, 6, total proteins of *P. larvae*-infected larvae (two different larvae); 5,7, total proteins of *P. larvae*-infected larvae (1/10 dilution) (two different larvae).

DISCUSSION

Metalloproteases have been described as virulence factors in a wide variety of micro-organisms, being involved in different mechanisms, including digestion of a wide variety of host proteins, attachment to host cells (Miyoshi & Shinoda, 2000), cytotoxicity (Kling *et al.*, 1997), and evasion of the immune system by degradation of antibacterial peptides (Casteels *et al.*, 1989).

Although the production of metalloproteases by *P. larvae* has been previously reported by several authors (Antúnez *et al.*, 2009; Dancer & Chantawannakul, 1997; Holst & Sturtevant, 1940; Hrabák & Martinek, 2007) none of these studies identified the genes that encode these proteins. The *P. larvae* genome is being assembled and automatically annotated; 4955 proteins have been reported and only two are described as extracellular proteases (GenBank accession numbers ZP_02326503.1 and ZP_02326602.1). However, there is a lack of experimental evidence about the function and localization of these proteins. In the present paper, and based on a partial nucleotide sequence of a gene encoding a *P. larvae* metalloprotease, we report the identification of an ORF in the *P. larvae* genome that encodes this metalloprotease. The amino acid sequence of the reported ORF showed 100 % identity with a *P. larvae* protein annotated as peptidase M4 thermolysin (GenBank accession number ZP_02330830.1). However, the coverage is only 78 %, since the first 116 amino acids have not been included in the annotation. This ORF also showed a high percentage identity (67 %) with the *P. larvae* extracellular proteases previously described (GenBank accession numbers ZP_02326503.1 and ZP_02326602.1), and with extracellular neutral proteases from *Paenibacillus polymyxa*, *Brevibacillus brevis* and *Bacillus megaterium* (GenBank accession numbers D00861.1, AP008955.1 and CP001983.1, respectively); in all these cases the coverage was 99−100 %. These results suggest that the identified sequence corresponds to a *P. larvae* metalloprotease.

The distribution of the gene that encodes this protein in *P. larvae* isolates of different geographical origins and belonging to different genotypes was assessed by PCR amplification. All isolates examined contained the gene, confirming that it is widely distributed.

The gene encoding *P. larvae* metalloprotease was overexpressed in *E. coli* and purified under denaturing conditions. A 59 kDa protein, corresponding to the predicted molecular mass of the preprometalloprotease, was obtained after purification. All extracellular bacterial neutral proteases are synthesized as inactive preproenzymes, consisting of a signal peptide, a prosequence and a mature sequence (Inouye *et al.*, 2007). The signal peptide acts as a signal for translocation of preproenzyme to the membrane. The preproenzyme is processed into the proenzyme by the signal peptidase. The proenzyme then acts as a molecular chaperone leading to an autocleavage of the peptide bond linking the pro and mature sequences (Inouye *et al.*, 2007). Some well-characterized examples are thermolysin and LasB, isolated from *Bacillus thermoproteolyticus* and *Pseudomonas aeruginosa*, respectively (Kooi *et al.*, 2005; McIver *et al.*, 1995; O'Donohue & Beaumont, 1996). It has been reported that is necessary that metalloproteases are expressed as preproenzymes, to allow the correct folding and catalytic procedure (Kooi *et al.*, 2005; McIver *et al.*, 1995; O'Donohue & Beaumont, 1996). As for *Bacillus thermoproteolyticus*, the *P. larvae* metalloprotease overexpressed in *E. coli* was obtained in inclusion bodies, and it should be purified to obtain the mature protein (Inouye *et al.*, 2007; Marie-Claire *et al.*, 1998). After storage of preprometalloprotease (59 kDa) at 4 °C the protein suffered autoproteolysis, giving a new protein of ~40 kDa.

Using the *P. larvae* recombinant metalloprotease generated in this study, a polyclonal mouse serum was produced. This antibody proved to be useful to identify the metalloprotease in the culture medium, showing that it is secreted outside the cells. This has been seen in the case of other insect pathogens such as *Photorhabdus luminescens*. This Gram-negative pathogen enters the haemocoel of

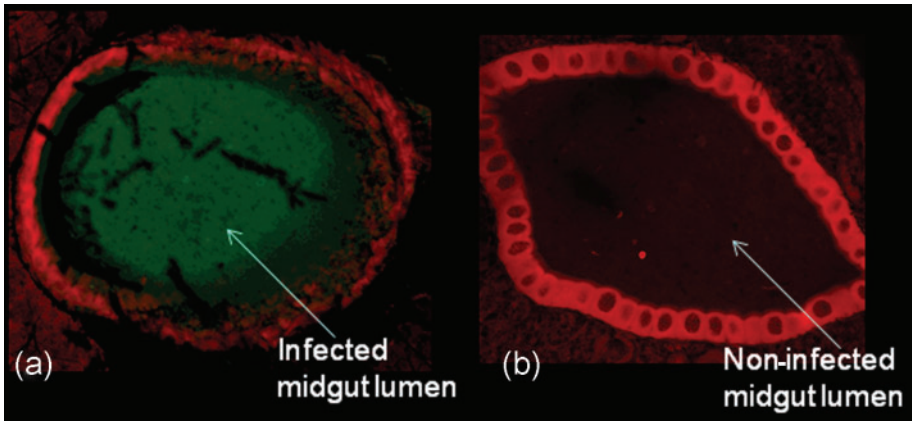

Fig. 5. Detection of *P. larvae* metalloprotease during infection of honeybee larvae. Larvae were infected with *P. larvae* spores (1000 spores per microlitre of larval food). Five days after infection, larvae were sacrificed, fixed with PFA and embedded in a gelatin/albumin matrix. Histological sections were prepared and subjected to immunofluorescence analysis (using a polyclonal antiserum raised against *P. larvae* metalloprotease and FITC-conjugated secondary antibody against mouse IgG) and FISH (using a eukaryotic probe conjugated to Cy3). Original images taken at ×40 magnification. The arrows indicate the presence of *P. larvae* metalloprotease. (a) *P. larvae*-infected larvae. (b) Non-infected larvae.

infected hosts, producing a number of secreted proteins that promote colonization and subsequent death of the insect (Held *et al.*, 2007).

The antiserum was also useful to confirm the expression of the metalloprotease by *P. larvae* vegetative cells and its presence on the spore surface. In some organisms, e.g. the fungal respiratory pathogen *Coccidioides posadasii*, expression of metalloproteases during spore differentiation has been reported (Hung *et al.*, 2005). The authors also reported that the enzyme cleaved surface antigens, preventing host recognition of endospores. The antiserum was shown to be specific for *P. larvae* metalloprotease since no other *P. larvae* or larval protein was recognized when it was used for Western blotting.

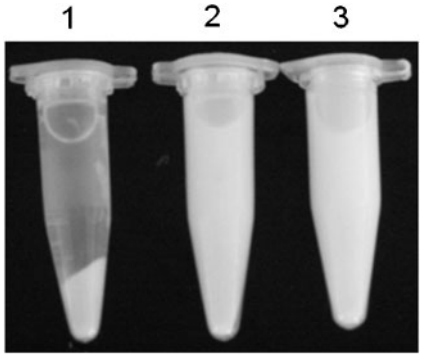

Fig. 6. Proteolytic activity of recombinant metalloprotease evaluated by the milk hydrolysis assay. 1, Recombinant protein; 2, negative control (protein elution buffer); 3, negative control (PBS).

When the anti-metalloprotease polyclonal mouse serum was used as the primary antibody in immunofluorescence-based microscopy assays, it was confirmed that the metalloprotease was expressed *in vivo* in the *P. larvae*-infected honeybee larval gut.

The images obtained using this approach also revealed clear damage of gut cells probably associated with the presence of bacteria expressing the metalloprotease, while the non-infected larvae showed a normal apparently healthy gut epithelium.

In conclusion, the present work reports the identification of a widespread *P. larvae* metalloprotease that is produced inside the cell, secreted to the external medium and could also be detected on the spore surface. It is produced *in vivo* during infection of honeybee larvae. This work contributes to the knowledge of the pathogenicity mechanisms of a bacterium of great economic significance and is one step in the characterization of potential *P. larvae* virulence factors.

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