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Characterization and Identification of Proteolytic Bacteria From the Gut of the Velvetbean Caterpillar (Lepidoptera: Noctuidae)

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ABSTRACT The characterization and identification of proteolytic bacteria from the gut of the velvetbean caterpillar (*Anticarsia gemmatalis*) were the objectives of this study. Twelve aerobic and anaerobic isolates of proteolytic bacteria were obtained from the caterpillar gut in calcium caseinate agar. The number of colony forming units (CFUs) of proteolytic bacteria was higher when the bacteria were extracted from caterpillars reared on artificial diet rather than on soybean leaves ($1.73 \pm 0.35 \times 10^3$ and $0.55 \pm 0.22 \times 10^3$ CFU/mg gut, respectively). The isolated bacteria were divided into five distinct groups, according to their polymerase chain reaction-restriction fragment-length polymorphism profiles. After molecular analysis, biochemical tests and fatty acid profile determination, the bacteria were identified as *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus gallinarum*, *Enterococcus mundtii*, and *Staphylococcus xylosus*. Bacterial proteolytic activity was assessed through in vitro colorimetric assays for (general) proteases, serine proteases, and cysteine proteases. The isolated bacteria were able of hydrolyzing all tested substrates, except *Staphylococcus xylosus*, which did not exhibit serine protease activity. This study provides support for the hypothesis that gut proteases from velvetbean caterpillar are not exclusively secreted by the insect cells but also by their symbiotic gut bacteria. The proteolytic activity from gut symbionts of the velvetbean caterpillar is suggestive of their potential role minimizing the potentially harmful consequences of protease inhibitors from some of this insect host plants, such as soybean, with implications for the management of this insect pest species.

KEY WORDS bacteria symbionts, proteases, 16S rRNA, protease inhibitor, pest control

Proteases are one of the main enzyme classes involved in the digestion of the velvetbean caterpillar *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), a key pest of soybean. Among digestive proteases, serine and cysteine proteases are the most studied and characterized, not only in *A. gemmatalis* (Oliveira et al. 2005, Xavier et al. 2005), but also in other insect species from different orders (Reeck et al. 1999, Terra and Ferreira 2005).

The use of protease inhibitors (PIs) as an alternative method for pest control is currently under intensive investigation (Pompermayer et al. 2001, Pilon et al. 2006). However, the velvetbean caterpillar is able to bypass the effects caused not only by natural inhibitors found in soybean plants (Soybean trypsin inhibitor [SBT] and Bowman-Birk-inhibitor [BBI]), but also by synthetic trypsin-like inhibitors such as benzamidine (Pilon et al. 2006). The digestive (trypsin-like) serine proteases purified from the velvetbean caterpillar are sensitive to inhibition by soybean protease inhibitors, as well as other serine protease inhibitors such as aprotinin and benzamidine (Oliveira et al. 2005, Xavier et al. 2005), but the caterpillars are still able to circumvent such host defense (Pilon et al. 2006, Fortunato et al. 2007). This versatility could arise from changes in protease expression in the caterpillars but also from enzymes synthesized by resident bacteria from their intestinal tract. Insect gut symbionts may contribute to host nutrition by producing enzymes, increasing digestion efficiency and supplying amino acids and vitamins (Cruden and Markovetz 1987, Brennan et al. 2004). However, the emphasis on most studies assessing the digestive role of insect gut bacteria is in carbohydrate (and sometimes lipid) degradation, rather than protein degradation, with few exceptions (Walker et al. 1999). In addition, little is known regarding the functional role of these microorganisms associated with Lepidoptera, despite recent advances with the diamondback moth (Yponomeutidae) and plant-promoting traits of its symbiont bacteria (Indiragandhi et al. 2007, 2008).

Gut bacteria from the velvetbean caterpillar may produce digestive proteases and, if so, such proteases may play a role in its adaptation to protease inhibitors, which have not yet received attention. Therefore, we

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reported here the isolation and characterization of gut bacteria from the velvetbean caterpillar. The proteolytic activity of the isolated gut bacteria was also determined aiming to provide preliminary evidence of their potential involvement on caterpillar protein digestion and possible role in allowing the adaptation of this insect pest species to protease inhibitors from its host plant.

Materials and Methods

Insects. The colony of the velvetbean caterpillar was established from eggs obtained from the laboratory colony maintained at the Soybean National Research Center of the Brazilian Agricultural Research Corporation (CNP Soja, EMBRAPA, Londrina, Brazil) on the same artificial diet used in our experiments (Hoffman-Campo et al. 1985). The insect eggs were surface-sterilized with UV light and subsequently transferred to plastic containers in a laminar flow chamber. After egg hatching, the caterpillars were place in plastic jars (500 ml; five caterpillars per jar), and four batches of five caterpillars each were reared on artificial diet throughout their development, whereas another four batches of five caterpillars were reared on soybean leaves (also throughout their development). The artificial diet used was protein rich and made of beans, soybean protein, casein, yeast, and wheat germ, enriched with ascorbic acid and complex B vitamins (Hoffman-Campo et al. 1985). The leaves used for rearing the caterpillars were obtained from previously unwounded soybean plants (*Glycine max* L. Merr. cultivar CAC1) at the V4 developmental stage, which were cultivated in greenhouse conditions (20–30°C), irrigated twice a day, and fertilized with N–P–K and Ca (50, 200, 100, and 100 ppm, respectively). The leaf petiole was involved in wet cotton when harvested for the caterpillars to retain humidity. Both diets were provided ad libitum to the respective insect batches. The caterpillars were reared in this two-diet system under controlled environmental conditions (25 ± 5°C, 70 ± 10% RH, and 14:10 [L:D] photoperiod) until reaching the fifth instar. All reagents were purchased from Sigma-Aldrich Química Brasil (São Paulo, Brazil), except ascorbic acid, which was purchased from Synth-LabSynth (São Paulo, Brazil). Beans, yeast, wheat germ, soybean protein, and vitamins were all obtained from the local market.

Isolation of Proteolytic Bacteria. Fifth-instar larvae reared either with diet or soybean leaves were collected and chilled at −18°C for 2 min to slow down metabolism. The insects were subsequently surface sterilized in 70% ethanol. To isolate anaerobic bacteria, three batches of five caterpillars each were dissected in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI), and their whole guts were inoculated in calcium caseinate agar. The petri dishes were maintained under CO_2:H_2 (95:5%) atmosphere at 30°C. To isolate aerobic bacteria, the caterpillars were dissected in a biosafety cabinet and also inoculated in calcium caseinate agar. All inoculations were performed in triplicate. After 48 h, colonies that formed clear zones caused by proteolysis were selected and inoculated in Luria-Bertaini agar until pure colonies were obtained.

Genomic DNA Extraction, Amplification, and Restriction Fragment-Length Polymorphism Analysis. The isolated proteolytic bacteria were inoculated in 3 ml of brain heart infusion (BHI) broth at 37°C for 16 h and centrifuged at 400*g*. The cultures were centrifuged at 13,400*g* for 5 min. The pellets were submitted to DNA extraction following Pospiech and Neumann (1995). These DNA samples were amplified with primers for the 16S rRNA: 5′-AGAGTTTGATCMTGG-3′ (16SF) and 5′-TACCTTGTTACGACTT-3′ (16SR), and the products were analyzed by polymerase chain reaction (PCR)-restriction fragment-length polymorphism (RFLP). The amplification reactions were performed in 25 μl, containing: 2.5 mM $MgCl_2$, *Taq* polymerase buffer 1×, 200 μM deoxyribonucleotides, 0.4 μM of each primer, 1 U of *Taq* polymerase, and 50 ng/μl DNA. The amplification program consisted of initial denaturing at 94°C for 2 min, followed by 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C), and extension (2 min at 72°C), followed by a final extension step (5 min at 72°C). A 25-μl aliquot was incubated with 5 U of *Hae*III and *Rsa*I restriction enzymes (Promega, Madison, WI). Based on their restriction profiles, different isolates were selected for identification and proteolytic activity evaluation.

Sequencing of the 16S rRNA and Sequence Analysis. The PCR products were extracted from the agarose gel (0.8%) with the QIAquick Gel Extraction Kit (Qiagen, Valencia CA), following the manufacturer's instructions. The DNA extraction from the gel was carried out to secure cleaner DNA fragments for the sequencing, free of dNTPs, primer remains, etc., which would be present if PCR amplicon were directly used. The amplicons were sequenced in a MegaBACE 500 equipment (GE Healthcare, São Paulo, Brazil) using the following 16S rRNA primers: 5′-AGAGTTTGATCMTGG-3′ (16SF) and 5′-TACCTTGTTACGACTT-3′ (16SR). The sequences were compared with the GenBank DNA sequence database, using the program BLASTn.

Biochemical Characterization of the Isolated Bacteria. The bacterial isolates were characterized by Gram staining and biochemical tests, according to bacteria identification protocols described by Sneath et al. (1986). Fatty acid methyl ester (FAME) analysis was performed to identify *Bacillus* species. The *Bacillus* were grown in trypticase soy agar (TSA) at 30°C for 24 h. FAMEs were extracted with the Instant FAME kit (MIDI, Newark, DE). Samples were analyzed by gas chromatography in an Agilent 7890 chromatographer. The results were compared with the ITSA 1.0 reference database.

Protein Determination and Enzyme Assays. Protein determination was performed following Bradford (1976), using bovine serum albumin (BSA) solutions as standards. Proteolytic bacteria were cultivated in 50 ml BHI at 37°C for 48 h at 400*g*. After incubation, the cultures were centrifuged at 13,400*g* for 10 min at 4°C.

Table 1. Total and proteolytic bacteria (no. CFUs ± SEM) found in the intestinal tract of *A. gemmatalis* reared with artificial diet or soybean leaves

Diet	Total bacteria (CFU/mg of gut tissue)		Proteolytic bacteria (CFU/mg of gut tissue)	
	Aerobiosis	Anaerobiosis	Aerobiosis	Anaerobiosis
Articial diet	$45.20 \pm 2.27 \times 10^3$	$5.19 \pm 0.32 \times 10^3$	$1.73 \pm 0.35 \times 10^3$	0.00 ± 0.00
Soybean leaves	$36.78 \pm 2.50 \times 10^3$	$3.00 \pm 0.23 \times 10^3$	$0.55 \pm 0.22 \times 10^3$	0.00 ± 0.00

The supernatants were collected and used in the enzyme assays. Gereral proteolytic activity was determined using 2% azocasein as substrate following Kunitz (1947), as adapted by Oliveira et al. (2005). Serine protease activity was determined following Erlanger et al. (1961) using the chromogenic substrate *N*-benzoyl-L-arginine *p*-nitroanilide (L-BApNA) to a final concentration of 0.5 mM. Cysteine protease activity was determined in the same conditions used for serine protease activity determination, but with the addition of 1 mM benzamidine, a serine protease inhibitor, and 5 mM dithiothreitol. All enzymatic assays were performed in triplicate.

Statistical Analyses. Results of colony forming units (CFUs) of gut bacteria isolated from the velvetbean caterpillar were subjected to two-way (2 diets × 2 incubation systems) analysis of variance (ANOVA; PROC GLM; SAS Institute 2002). Results of enzyme activity of the five bacterial isolates were subjected to ANOVAs followed by Tukey's honestly significant difference (HSD) test ($P < 0.05$), when appropriate (PROC GLM; SAS Institute 2002). Assumptions of normality and homogeneity of variance were checked (PROC UNIVARIATE; SAS Institute 2002), but no data transformation was necessary.

Results

Isolation and Colony Formation of Gut Bacteria. The results of number of CFUs of gut bacteria obtained from velvetbean caterpillars are shown in Table 1. The ANOVA for CFUs of gut bacteria indicated that caterpillar rearing diet (artificial diet versus soybean leaves) significantly affected the CFUs of both total ($F_{1,11} = 9.70, P = 0.01$) and proteolytic bacteria ($F_{1,4} = 7.00$, $P = 0.04$), with significantly more colonies formed when the bacteria were extracted from caterpillars reared on artificial diet ($25.93 \pm 9.01 \times 10^3$ and $1.73 \pm 0.35 \times 10^3$ CFU/mg gut for total and proteolytic bacteria, respectively) compared with bacteria from caterpillars reared on soybean leaves ($19.89 \pm 7.36 \times 10^3$ and $0.55 \pm 0.22 \times 10^3$ CFU/mg gut for total and proteolytic bacteria, respectively). Bacteria colony formation was ~10 times higher under aerobic condition ($40.99 \pm 4.21 \times 10^3$ CFU/mg gut) than under anaerobic conditions ($4.09 \pm 0.52 \times 10^3$ CFU/mg gut) for total bacteria ($F_{1,11} = 469.22$, $P < 0.0001$). There was no significant interaction between caterpillar diet and aerobiosis/anaerobiosis for total bacteria isolated from the caterpillar gut ($F_{1,11} = 3.35$, $P = 0.10$). No proteolytic bacteria were detected under anaerobic conditions. Twelve morphologically distinct proteolytic bacteria were isolated—seven from the gut of caterpillars reared on artificial diet and five from the gut of caterpillars reared on soybean leaves.

RFLP Analysis of PCR-Amplified 16S rRNA. The results of the PCR-RFLP analysis are shown in Fig. 1. Figure 1A shows the DNA samples after cleavage with the restriction enzyme *Hae*III. Samples with the same restriction profile were cleaved with the enzyme *Rsa*I to ensure that these bacteria pertain to the same group (Fig. 1B). Five distinct groups were found, based on the different restriction profiles: group 1 (isolate 1), group 2 (isolates 2, 5, and 7), group 3 (isolates 3, 8, and 11), group 4 (isolates 4 and 6), and group 5 (isolates 9, 10, and 12). Based on these results, one isolate from each group was selected for further characterization.

Sequence Analysis of the 16S rRNA, Biochemical Characterization, and Identification. Isolate identification was performed by sequencing the 16S rRNA gene of one member of each group of bacterial isolates. The PCR products generated sequences of ≈1,400 bp. Molecular analysis allowed the identification of all bacteria (Table 2). Molecular identification was confirmed based on results from morphological, biochemical (Table 3), and membrane fatty acid analyses. Isolates from groups 1 and 2 were respectively identified as *Bacillus subtilis* and *Bacillus cereus*, based on their fatty acid profile (data not shown) and on the following observed parameters: rod-shaped morphology, aerobiosis, mobility, and catalase positivity. Groups 3 and 4 were identified as group D *Streptococcus* (*Enterococcus*) with the shape of gram-positive diplococci. These isolates were able to perform esculin hydrolysis in the presence of 40% bile and to grow in 6.5% NaCl. Finally, motility and pigmentation tests allowed isolates from groups 3 and 4 to be identified as *Enterococcus mundtti* and *Enterococcus gallinarum*, respectively. The isolate from group 5 exhibited characteristics of staphylococci: shape of irregularly grouped cocci, positive for catalase, negative for oxidase, and resistance to bacitracin. Furthermore, this isolate was identified as a member of *Staphylococcus* sp. group because of its negativity for coagulase, resistance to novobiocin, and acid production from xylosis, which finally identified this isolate as *S. xylosus.*

Enzymatic Assays. Total protease, serine protease, and cysteine protease activities were determined in the supernatants from bacterial cultures and are depicted in Fig. 2. ANOVA performed to compare the proteolytic activity of the different bacteria isolates indicated significant differences among them for all tested substrates (general proteases: $F_{4,10} = 8.46, P = 0.003$; serine proteases: $F_{4,10} = 20.76$, $P < 0.0001$; cysteine protease: $F_{4,10} = 6.65$, $P =$

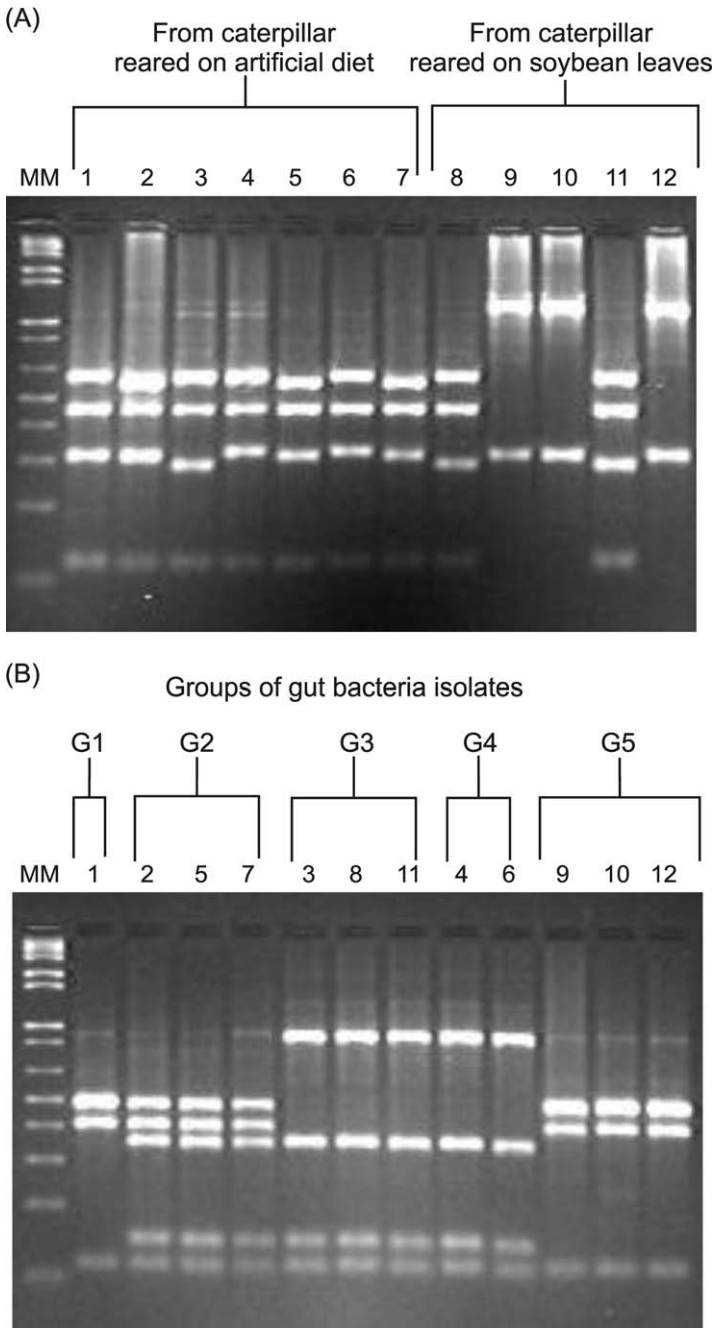

Fig. 1. PCR-RFLP analysis of the 16S rRNA gene of bacteria isolated from the gut of the velvebean caterpillar (*A. gemmatalis*). The restriction profiles were obtained after sample digestion with the restriction enzymes *Hae*III (A) and *Rsa*I (B). Lane MM contains the molecular mass standards, and lanes 1–12 refer to each proteolytic isolate. The isolates obtained from caterpillars reared on each diet are indicated in A, whereas the isolate groups (G1, G2, G3, G4, G5) are indicated in B.

0.007). The bacterial species isolated were capable of hydrolyzing all substrates, except *S. xylosus*, which did not exhibit serine protease activity. *E. mundtti* exhibited the highest proteolytic activity, whereas *B. cereus* exhibited the highest serine and cysteine protease activities.

Discussion

Insect gut microbiota is greatly influenced by insect diet (Walker et al. 1999). Bacteria found in the artificial diet and in soybean leaves are ingested by *A. gemmatalis*, and the finding of bacterial symbionts

Table 2. Identification of the bacterial groups isolated from the gut of the velvetbean caterpillar (*A. gemmatalis*) based on the 16S rRNA gene

Molecular analysis	Bacterial groups				
	1	2	3	4	5
Number of sequenced bases	1,419	329	1,337	1,408	1,397
Similarity (%)	97%	91%	99%	99%	99%
GenBank accession number	EU569316	EU569317	EU569314	EU569315	EU569313
Identification	*Bacillus subtilis*	*Bacillus cereus*	*Enterococcus mundtii*	*Enterococcus gallinarum*	*Staphylococcus xylosus*

associated with the gut of this insect species is expected. Although we do not have direct evidence that the isolated bacteria originated from the insect diet, such a possibility is very high because potential contamination from other sources was minimized during the bacterial isolation. Our results indicate significant differences in CFUs of gut bacteria from caterpillars reared on artificial diet (higher) and soybean leaves (lower). This is probably because of the higher protein content present in the artificial diet, which favors the development of proteolytic bacteria.

The gut bacteria isolated from the velvetbean caterpillar were mainly aerobic, particularly the proteolytic bacteria, although the low levels of O_2 encountered in the insect gut allow the survival of facultative anaerobic bacteria. However, as the intestinal tract of *A. gemmatalis* exhibits a relatively simple compartimentalization, its microenvironment is probably not fully anoxic because of a higher O_2 flow potentially allowing the development of both aerobic and facultative anaerobic bacteria. This is a contrast with the termite gut, which exhibits a prevailing anoxic atmosphere favoring the development of anaerobic microorganisms (Brune and Friedrich 2000). Although the prevalence of aerobic and facultative anaerobic bacteria in the caterpillar gut is plausible, the culture medium used in our study may also lack nutritional compounds required for the growth of the anaerobic gut bacteria explaining the lower numbers of their CFUs. In addition, our culture media, which is suitable for detection of proteolytic bacteria, does not

Table 3. Morphological and biochemical properties of the bacterial groups isolated from the gut of the velvetbean caterpillar (*A. gemmatalis*)

Traits	Bacterial groups				
	1	2	3	4	5
Morphology	Rod	Rod	Cocci	Cocci	Cocci
Gram staining	+	+	+	+	+
Arrangement	Single	Single	Diplococci	Diplococci	Groups
Motility	+	+	−	+	−
Oxidation	−	−	−	−	−
Catalase test	+	+	−	−	+
Coagulase test	−	−	−	+	−
Hemolysis	β	β	α	α	α
Camp test	ND	ND	−	−	−
NaCl 6.5% tolerance	+	+	+	+	+
Esculine hydrolysis	+	−	+	+	−
MacConkey growth	+	−	−	−	−
Pigment on tryptic soy agar	ND	ND	+	−	ND
Acid production of carbohydrates					
L-arabinose	+	+	+	+	+
Cellobiose	+	−	−	−	−
Fructose	+	+	+	+	+
Glucose	+	+	+	+	+
Lactose	−	−	+	+	−
Maltose	−	+	−	+	+
D-mannitol	+	−	+	+	+
D-mannose	−	−	−	−	+
D-melibiose	+	+	+	+	+
Raffinose	+	+	−	+	−
Ribose	−	−	+	+	−
Sorbitol	ND	ND	+	+	ND
Sorbose	ND	ND	−	−	ND
Sucrose	+	+	+	+	+
D-trehalose	+	+	+	+	+
D-xylose	−	−	+	−	+
Bacitracin (0.04 U)	ND	ND	R	R	R
Novobiocin (5 μg)	ND	ND	ND	ND	R
Vancomycin (30 μg)	ND	ND	S	S	S
Identification	*Bacillus subtilis*	*Bacillus cereus*	*Enterococcus mundtii*	*Enterococcus gallinarum*	*Staphylococcus xylosus*

ND, not determined; R, resistant; S, susceptible.

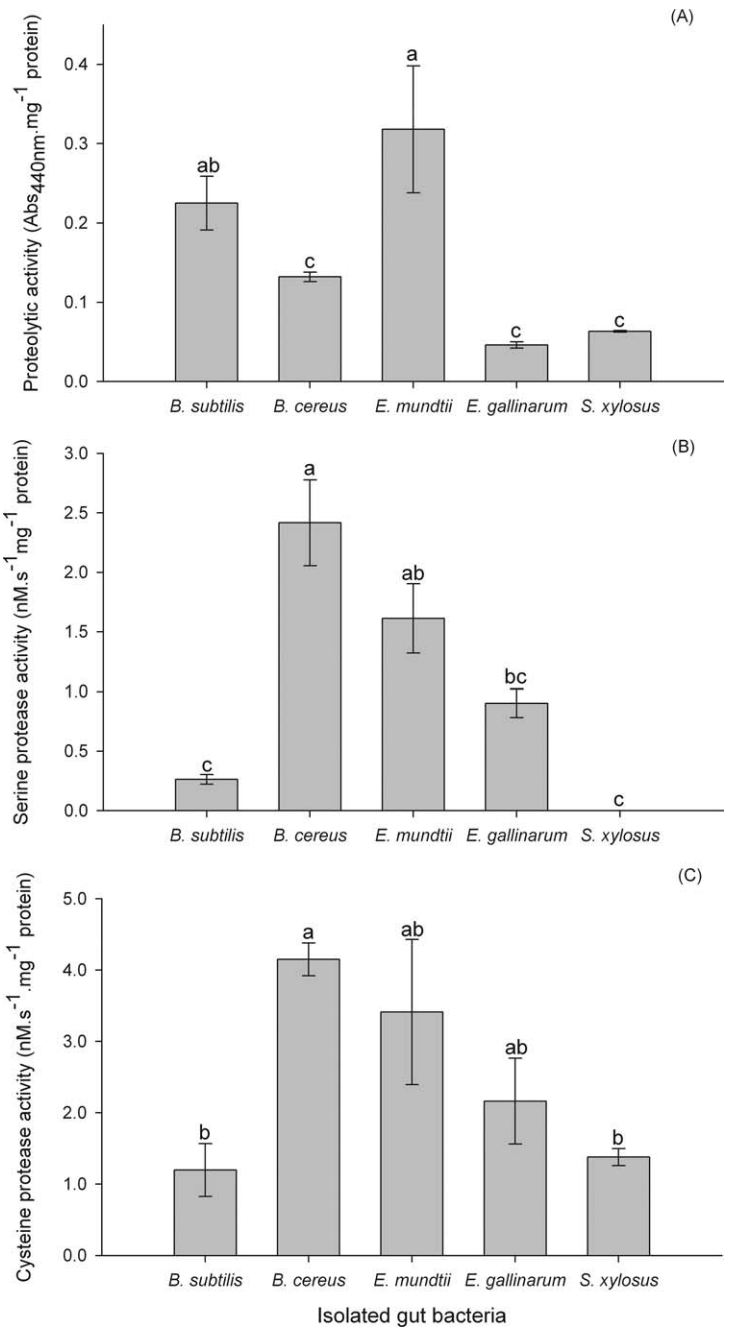

Fig. 2. Specific activity of general proteases (A), serine proteases (B), and cysteine proteases (C) determined in the supernatants of bacterial cultures. Each histogram bar represents the mean of three replicates. The vertical bars represent SEM. Histogram bars with the same letter are not significantly different by Tukey's HSD test ($P < 0.05$).

allow assessing of the entire cultivable bacteria community.

The morphological and biochemical characterizations of the isolated gut bacteria from the velvetbean caterpillar, together with the PCR-RFLP analysis, showed the presence of only five different species of proteolytic bacteria. Among the bacteria isolated, *Bacillus* is the genus most commonly found in insect guts (Wenzel et al. 2002, Ertürk and Demirbag 2006), and some *Bacillus* species are pathogenic to insects and used as biological control agents of insect pests (Ertürk and Demirbag 2006). *Enterococcus* has also been reported in the gut of fruit flies (Kuzina et al. 2001), houseflies (Macovei and Zurek 2006), beetles (Schloss

et al. 2006), and ants (Li et al. 2005). In contrast, *Staphylococcus* is not so frequently isolated from insect guts as *Bacillus* and *Enterococcus.* No gram-negative proteolytic bacteria were isolated from the gut of *A. gemmatalis*, unliked *Cydia pomonella* L. (Lepidoptera: Tortricidae) (Ertürk and Demirbag 2006).

The gut bacteria isolated from caterpillars of *A. gemmatalis* were able of hydrolyzing azocasein and L-BApNA in the presence of benzamidine, showing cysteine protease activity. In addition, all isolates exhibited serine protease activity, except *S. xylosus. B. cereus* showed the highest activity of serine and cysteine proteases, which is not a surprise because most of the commercially available serine proteases are produced by microorganisms from the genus *Bacillus* (Olajuyigbe and Ajele 2005).

Our data indicate that proteases found in the gut of *A. gemmatalis* are produced both by the insect and by part of the resident microbiota. Besides of providing an important contribution to the understanding of the insect–microorganism interaction, our results suggest a potential role of these gut bacteria from velvetbean caterpillars in allowing the adaptation of this insect pest species to hosts rich in protease inhibitors, such as soybean. However, the purification and characterization of the gut bacteria protease are still necessary to recognize if indeed they are not inhibited by the soybean protease inhibitors (unlike the gut proteases of the velvetbean caterpillars) and may therefore minimize the effect of such inhibitors in caterpillars of *A. gemmatalis.* This information has pest management implications because it may allow the recognition of the host range of this insect species, is likely to be helpful in defining suitable mimetic analogs of protease inhibitors for use as bioinsecticides, and may also be helpful in guiding soybean breeding programs in developing varieties resistant to its main pest species.

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