

Article

Cellulolytic Bacteria Associated with the Gut of *Dendroctonus armandi* Larvae (Coleoptera: Curculionidae: Scolytinae)

Xia Hu, Jiamin Yu, Chunyan Wang and Hui Chen *

College of Forestry, Northwest A&F University, Yangling 712100, Shaanxi, China;

E-Mails: lake-autumn@163.com (X.H.); yjm407@nwsuaf.edu.cn (J.Y.); chunyan@nwsuaf.edu.cn (C.W.)

* Author to whom correspondence should be addressed; E-Mail: chenhui@nwsuaf.edu.cn;

Tel./Fax: +86-29-8708-2083.

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Abstract: The object of this study was to investigate the cellulolytic bacterial community in the intestine of the Chinese white pine beetle (*Dendroctonus armandi*) larvae. A total of 91 cellulolytic bacteria were isolated and assigned to 11 genotypes using amplified ribosomal DNA restriction analysis (ARDRA). Partial 16S rDNA sequence analysis and morphological tests were used to assign the 11 representative isolates. The results showed that the isolates belonged to α -Proteobacteria, γ -Proteobacteria and Firmicutes. Members of γ -Proteobacteria were the most frequently represented species and accounted for 73.6% of all the cellulolytic bacteria. The majority of cellulolytic bacteria in *D. armandi* larva gut were identified as *Serratia* and accounted for 49.5%, followed by *Pseudomonas*, which accounted for 22%. In addition, members of *Bacillus*, *Brevundimonas*, *Paenibacillus*, *Pseudoxanthomonas*, *Methylobacterium* and *Sphingomonas* were found in the *D. armandi* larva gut. *Brevundimonas kwangchunensis*, *Brevundimonas vesicularis*, *Methylobacterium populi* and *Pseudoxanthomonas mexicana* were reported to be cellulolytic for the first time in this study. Information generated from the present study might contribute towards understanding the relationship between bark beetle and its gut flora.

Keywords: gut microflora; ARDRA; *Dendroctonus armandi*; cellulase; bark beetle

1. Introduction

As the primary structural material of plant cell walls, cellulose is a complex carbohydrate polymer of glucose residues that are connected by β -1,4 linkages [1]. In nature, lignocellulose is degraded by hydrolytic and oxidative enzymes that are produced mainly by fungi and bacteria which are able to synergistically degrade cellulose, hemicellulose and lignin [2]. Some insects, such as termites, beetles, wood-feeding roaches and leaf-cutting ants, can use lignocellulosic substrates as their main food source and are highly efficient at degrading cellulose to glucose as an energy source [3]. The gut systems of these insects are diverse, highly adapted and considered to be highly efficient natural bioreactors [3]. Furthermore, the intestinal microorganisms of these lignocellulose-degrading insects are considered to be essential for cellulose digestion [4]. Biodegradation of cellulose and hemicellulose, which are the recalcitrant components of the plant cell wall, by the cellulolytic activity of endosymbionts in the digestive tract of insects such as termites has been well studied [5]. However, the role of the symbiotic intestinal microbes of bark beetles, which is one of the most important woody plant-feeding insects, is unclear.

Bark beetles, especially *Dendroctonus* species, are considered to be serious pests of coniferous forests [6]. The Chinese white pine beetle (*Dendroctonus armandi* Tsai and Li), which is an important pest in the Qinling and Bashan Mountains in Northern China, can kill living *Pinus armandi* and has brought about huge economic losses; therefore, it represents a major disturbance factor for the ecosystem [7]. The larvae of *D. armandi* bore into the bark of *P. armandi* and feed on its nutrient-poor phloem substrates. Symbiotic microbes provide nutritional supplements that benefit their hosts [8], and the intestinal bacteria in the hindgut of bark beetles, such as *D. frontalis* and *D. rhizophagus*, might be responsible for the cellulose degradation [9,10].

Our previous work has investigated the gut bacteria community of *D. armandi* using uncultured methods [11]. Because little is known regarding the potential roles that gut bacteria play in its cellulose digestion, this work attempted to investigate the cellulose-degrading bacterial community in the gut of *D. armandi* larvae using amplified ribosomal DNA restriction analysis (ARDRA), which could specifically evaluate the nutritional contributions of gut bacteria to the Chinese white pine beetle.

2. Experimental Section

2.1. Insect Collection and Dissection

D. armandi larvae were collected from the bark of infested *P. armandi* at the Huoditang Experimental Forest Station of Northwest A&F University, which is located on the southern slope of the middle Qinling Mountains (33°18′–33°28′ N, 108°21′–108°39′ E), Shaanxi, CN, in August 2012. All samples were obtained manually and directly from galleries of infested pine trees using fine forceps and then transported to the laboratory in sterile vials containing sterile moist paper, where they were left for 48 h to allow the indigested food to leave the intestinal canal. Nine healthy larvae were sterilized by submersion in 70% ethanol for 3 min and then rinsed in sterile water twice prior to dissection. Specimens were dissected under a stereomicroscope using insect pins to obtain the hindguts and then transferred to 1.5-mL microcentrifuge tubes containing 0.5 mL of PBS.

2.2. Isolation and Counting of Cellulolytic Microorganisms

For viable counts, three tubes, each with an individual gut, were squeezed several times using a plastic pestle, 1 mL of PBS was added, and the sample was centrifuged at 4000 rpm at 4 °C for 10 s to separate the microbial cells from the gut wall. The bacterial suspension was serially diluted ten-fold (to 10^{-9}). One hundred microliters of each dilution was spread on plates containing solid medium 1. Only the colonies encircled by a clear zone after Congo red (1 mg/mL) staining were counted. The dilution series was also used for enrichment in 50-mL flasks containing 10 mL of medium 1 [peptone, 5 g/L; yeast extract, 0.1 g/L; K_2HPO_4 , 1 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; carboxymethyl cellulose sodium salt (CMC, low viscosity; Sigma, St. Louis, MO, USA), 10 g/L; Na_2CO_3 , 10 g/L (sterilized separately); pH 7.0]. The flasks were incubated aerobically and shaken at 180 rpm at 28 °C in the dark. After 1 month, the cellulolytic colonies were counted as described above.

To isolate cellulolytic bacteria, methods and media were adapted from Delalibera *et al.* [9]. In brief, the supernatant (containing bacteria) of a larval gut was transferred to a 50-mL flask containing 15 mL medium 2a, 5 g/L filter paper strips (Whatman), 40 mg/L Bacto yeast extract (Becton Dickinson, Sparks, MD), 100 mg/L malt extract (Becton Dickinson) and 2 g/L $CaCO_3$, pH 7.0 or medium 2b, which contained 5 g/L CMC, 30 mg/L yeast extract, 100 mg/L malt extract, and 2 g/L $CaCO_3$, pH 7.0. Liquid incubation was performed as described above. To confirm crystalline cellulose, microbes with carboxymethylcellulase activity were inoculated in medium 2c, which contained 5 g/L phosphoric- acid-swollen Avicel (PH105), 1.9 g/L K_2HPO_3 , 0.94 g/L KH_2PO_3 , 1.68 g/L $NaHCO_3$, 1.6 g/L KCl, 1.43 g/L NaCl, 0.15 g/L NH_4Cl , 0.037 g/L $7H_2O \cdot MgSO_4$, 0.017 g/L $CaCl_2 \cdot 2H_2O$ and 0.1 g/L yeast extract, pH 7.0. After 7 days of enrichment, 100 μ L serial dilutions of the cultures were transferred to solid medium 3 (5 g/L CMC, 0.2 g/L yeast extract, and 12 g/L agar, pH 7.0). All experiments were performed in triplicate.

2.3. CMC Assay

Microorganisms on solid media 2a and media 2b were tested for their ability to degrade CMC by covering the Petri dishes with Congo red dye [12]. CMC degradation was indicated by a clear zone surrounding the colonies.

2.4. ARDRA

Bacterial DNA was extracted using the E.Z.N.A. bacteria DNA kit (Omega Biotech, Doraville, GA, USA) according to the manufacturer's directions. The 16S rRNA bacterial genes were amplified using primers fD1 and rP1 [13], and the amplified DNA was for restriction digestion.

Restriction of 5 μ L PCR products was carried out for 2 h at 37 °C using 1.5 U of each of the following restriction enzymes: *Hha*I, *Afa*I and *Msp*I (TaKaRa Biotechnology, Dalian, China). Aliquots (5 μ L) of each digested product were analyzed using gel electrophoresis in an 8% nondenaturing acrylamide gel (acrylamide: *N,N'*-methylenebisacrylamide, 29:1) [14] by staining with ethidium bromide solution and photographed under UV light using the Gel DocTMXR System (Bio-Rad, Hercules, CA, USA). For each distinct ARDRA group, one bacterial isolate was selected for sequencing and morphological characterization.

2.5. Molecular and Phylogenetic Analysis

Sequences were matched to sequences in the RDP II database [15], and BLASTed to the NCBI database [16] to select reliable, highly similar sequences to determine the classification status of these bacteria. All sequences were deposited into the NCBI database with accession numbers (KF501473-KF501484). The phylogenetic relationships of these intestinal bacteria were analyzed using molecular phylogeny. The sequences were aligned using MUSCLE [17], which is available in MEGA 5, and a Neighbor-joining tree was constructed [18]. To calculate the support for each clade, bootstrap analysis was performed with 1000 replications.

2.6. Operational Taxonomic Units (OTU)

Sequences in the phylogenetic tree were formatted as FASTA files and used to construct distance matrices for gene fragment sequences using MOTHUR 1.29.0. The distance matrices were used as the input files to define the OTU on the basis of a similarity distance cutoff of 0.03; this distance generally corresponded to a species [19]. Sequences belonging to the same OTU_{0.03} cluster were circumscribed with brackets in the phylogenetic trees and were identified using I-VIII for the purpose of clarity.

2.7. Morphological and Physiological Characteristic Tests

For each ARDRA group, one representative isolate was selected for microscopic examination. Cell morphology was examined using light microscopy (Model ZS2-ILST; Olympus, Tokyo, Japan). Gram staining was carried out using the standard Gram reaction combined with the KOH lysis test [20]. Flagella were stained according to the method of Leifsson [21].

3. Results and Discussion

Viable counting demonstrated that the population density of the cellulolytic bacteria in *D. armandi* larvae was $1.1 \pm 0.46 \times 10^2$ CFU/gut, which was lower than that of other beetles, such as *Saperda vestita* (2.4×10^5 to 3.57×10^6 CFU/gut) [9] and *D. rhizophagus* ($2.3 \pm 0.43 \times 10^3$ CFU/gut) [10]. After one month of enrichment, the population density of the bacteria with CMCase activities was $1.5 \pm 0.4 \times 10^4$ CFU/gut, which was also lower than that of other insects, such as *Holotrichia parallela* ($1.14 \pm 0.13 \times 10^8$ CFU/gut) and *Pachnoda marginata* ($2.5 \pm 1.1 \times 10^8$ CFU/mLgut) [4,22]. These findings support similar findings from our earlier study [11] that suggested that the bacterial community in *D. armandi* larvae has low abundance and complexity compared to those of other beetles.

Eleven cellulolytic bacteria that were directly from the gut content and had the same colony characters were detected on the dilution plates, while, after one-month incubation, 91 cellulolytic bacteria were isolated. Of these, 32 were obtained using the enriching method on filter paper (2a), and 59 isolates were obtained by direct plating on CMC medium (2b). These isolates produced variable zones of CMC clearance (Figure 1) with a ratio of the clearance zone diameter (mm) to the colony diameter ranging from 1.0 to 6.6. Eleven direct isolates and 91 enrichment isolates were assigned to 11 genotypes using ARDRA. Combining the results of molecular and phylogenetic analysis (Figure 2) and morphological identification, 11 direct isolates belonged to *Serratia* of Proteobacteria, and the

genotype was the same as the representative strain B (Table 1). The gut associated cellulolytic enrichment bacteria in *D. armandi* larvae were clustered into 2 phyla (Proteobacteria and Firmicutes), and represented 8 different genera (Figure 2). The cellulolytic bacterial communities were represented by members of the Proteobacteria (82.4%) and Firmicutes (17.6%) phyla (Figure 3). On the generic level, *Serratia* (49.5%), *Pseudomonas* (22%) and *Bacillus* (13.2%) were the dominant identified genera, with 45, 20 and 12 isolates, respectively. The remaining bacterial isolates composed 15.4% *Brevundimonas* (5.5%), *Paenibacillus* (4.4%), *Pseudoxanthomonas* (2.2%), *Methylobacterium* (2.2%) and *Sphingomonas* (1.1%).

Figure 1. Screening for cellulolytic bacteria by covering the Petri dishes with Congo red dye. A zone of clearance surrounding a colony is indicative of carboxymethyl cellulose (CMC) hydrolysis by secreted CMCase.

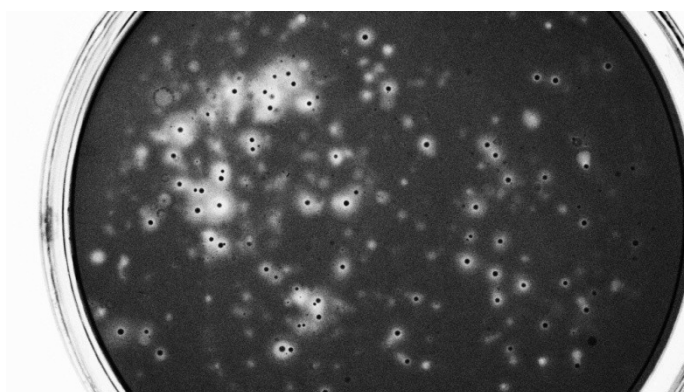


Figure 2. Neighbor-joining phylogenetic tree of 16S rRNA gene fragments of *Dendroctonus armandi* gut cellulolytic bacteria (enrichment isolates) (model: Tajima-Nei+G).

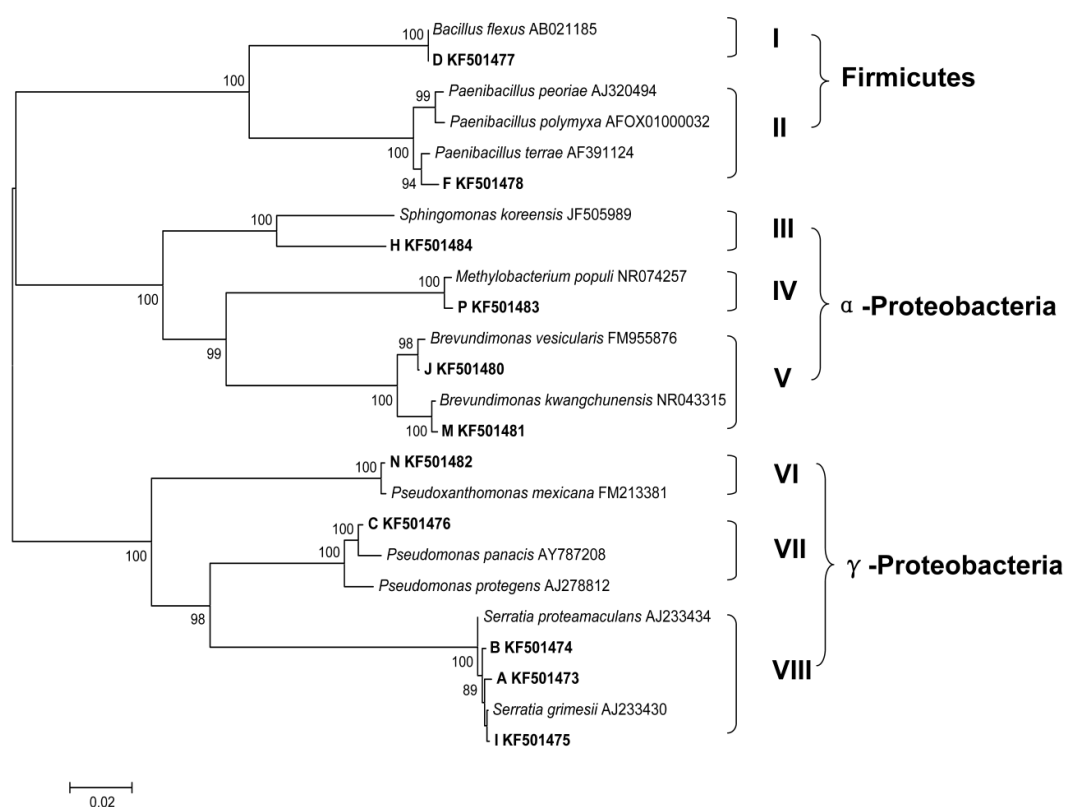
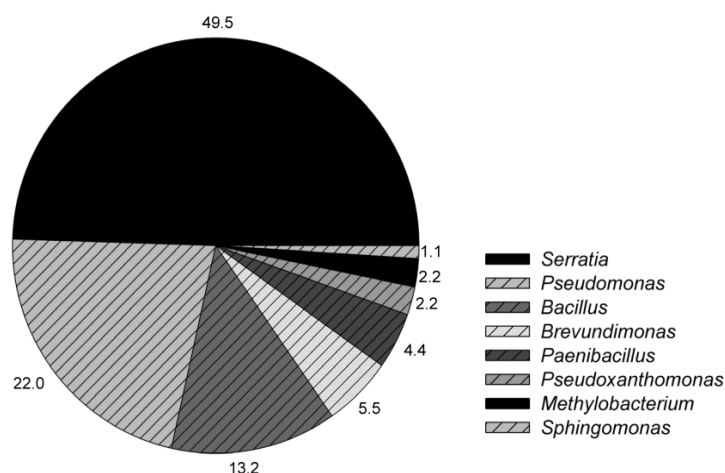


Table 1. Characteristics of cellulose-degrading microorganisms that were separated from guts of *D. armandi*. +, Positive; −, Negative; ND, no data available; * G, Gram staining.

Representative strains	Closest known taxa	Colony	Cell morphology	Pellicle	Flagella	Spore	G *	Numero of isolates	
								Medium 2a	Medium 2b
A	<i>Serratia</i>	Pink	Rods, $0.39\text{--}0.72 \times 0.92\text{--}6.43 \mu\text{m}$	+	Peritrichous	−	−	2	5
B	<i>Serratia</i>	Pink	Rods, $0.31\text{--}0.66 \times 0.96\text{--}5.54 \mu\text{m}$	+	Peritrichous	−	−	11	20
C	<i>Pseudomonas</i>	Ivory	Rods, $0.35\text{--}0.66 \times 1.08\text{--}2.75 \mu\text{m}$	−	ND	−	−	10	10
D	<i>Bacillus</i>	Ivory	Rods, $0.38\text{--}0.67 \times 0.84\text{--}3.24 \mu\text{m}$	+	Peritrichous	+	+	2	10
F	<i>Paenibacillus</i>	Pink	Rods, $0.64\text{--}1.32 \times 2.12\text{--}6.10 \mu\text{m}$	−	Peritrichous	+	+	0	4
H	<i>Sphingomonas</i>	Yellow	Rods, $0.32\text{--}0.59 \times 0.98\text{--}10.62 \mu\text{m}$	−	Peritrichous	+	−	0	1
I	<i>Serratia</i>	Pink	Rods, $0.36\text{--}0.70 \times 0.91\text{--}4.98 \mu\text{m}$	+	Peritrichous	−	−	2	5
J	<i>Brevundimonas</i>	Pale yellow	Rods, $0.61\text{--}1.13 \times 1.64\text{--}3.50 \mu\text{m}$	−	Peritrichous	+	−	0	2
M	<i>Brevundimonas</i>	Ivory	Rods, $0.28\text{--}0.50 \times 1.16\text{--}3.40 \mu\text{m}$	−	Peritrichous	+	−	2	1
N	<i>Pseudoxanthomonas</i>	Yellow	Rods, $0.21\text{--}0.38 \times 1.05\text{--}2.50 \mu\text{m}$	+	Polar	−	−	1	1
P	<i>Methylobacterium</i>	Pale yellow	Rods, $0.32\text{--}0.50 \times 0.89\text{--}2.40 \mu\text{m}$	−	Polar or subpolar	−	−	2	0

Figure 3. Bacterial groups derived from *Dendroctonus armandi* 16S rRNA genes ($n = 91$).

According to distance matrices of the sequences, 8 OTUs_{0.03} clusters were obtained and separately marked as I-VIII. Cluster VIII, which was related to *Serratia* was the most dominant group in the larval gut according to the results of phylogenetic analysis and morphological identification. Found widespread around the globe, *Serratia* is a Gram-negative bacterium that is capable of thriving in diverse environments that include water, soil and the digestive tracts of various animals [23]. *Serratia* spp. have been previously isolated or detected in *Dendroctonus* spp. [10,24,25]. Known for the ability to produce a myriad of extracellular enzymes such as cellulases [26], *Serratia* spp. can help the host wood-feeding insect to digest its food better. In addition, the impact of *Serratia* species on other organisms ranged from parasitic to symbiotic, and these bacteria have in common the ability to resist attack, respond appropriately to environmental conditions and outcompete other microorganisms when colonizing their respective niches [23]. Above all, *Serratia* was widespread, competitive and closely related to its host insect, resulting in the high abundance of *Serratia* spp.

Pseudomonas was the second-most dominant group of the cellulolytic bacterial community in the gut of *D. armandi* larvae. The dominance of *Pseudomonas* was also present in other Coleoptera insect larvae [4]. Bacteria of the genus *Pseudomonas* can be found in many different environments, including plant, animal tissue, water and soil, and they have the ability to metabolize a variety of nutrients [27]. Many *Pseudomonas* species are opportunistic pathogens that infect humans, animals and plants [28–30], but some *Pseudomonas* species have been reported to degrade cellulose [31,32].

Bacillus was the third-most dominant group of the cellulolytic bacterial community in the gut of *D. armandi* larvae. The cellulolytic activity of *Bacillus flexus* had been well described [33].

The cellulolytic activities of the other bacterial groups that were found in this study have also been reported previously. The cellulolytic activity of *Paenibacillus* was reported [34], and Okeke and Lu reported that *Pseudoxanthomonas* produced an array of cellulolytic-xylanolytic enzymes (filter paper cellulase, β -glucosidase, xylanase and β -xylosidase) and also assayed these activities [35]. *Methylobacterium* expressing β -1,4-endoglucanase was described [36], and an uncultured cellulolytic strain that is closely related to *Sphingomonas* was also found in the gut of the termite *Zootermopsis angusticollis* (Hagen) [37].

To the best of our knowledge, this is the first report describing *Brevundimonas kwangchunensis*, *Brevundimonas vesicularis*, *Methylobacterium populi* and *Pseudoxanthomonas mexicana* as being

cellulolytic. The ratio of the CMC clearance zone diameter to the colony diameter ranged from 4.1 to 5.2 for *B. kwangchunensis*, indicating robust CMCase production. These cellulolytic bacterial isolates exhibited great potential for the study of novel enzymes in cellulose degradation and for improving the bioconversion of lignocellulosic biomass. Cellulolytic bacteria have also been isolated from the larval gut of the bark beetles *Ips pini* and *Dendroctonus frontalis* [9]. All isolates were active against carboxymethylcellulose; nevertheless, only a *S. vestita* isolate showed activity against filter paper. A study of the bacterial flora in the guts of *Dendroctonus rhizophagus* proposed that most of the bacterial genera that were present could be implicated in nitrogen fixation and cellulose breakdown, which are important roles associated with insect development and fitness, particularly given the challenging environment that is inhabited by bark beetles [10]. All of these results indicate that the bacteria in beetle hindguts play an important role in the degradation of plants and other organic matters that are consumed by *D. armandi* larvae.

4. Conclusions

This study investigated the structure of the gut-associated, cellulolytic bacterial communities in *D. armandi* larvae that were collected from the southern slope of the middle Qinling Mountains in August 2012. Using direct dilution, 11 cellulolytic bacterial stains were identified to be of one genotype that was related to genus *Serratia*. Additionally, seven genera with varied cellulolytic activities were observed after enrichment. Moreover, some bacterial species were reported to be cellulolytic for the first time in this study, which demonstrates that the bark beetle gut has a great potential to be a source of novel cellulolytic microorganisms.

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Author Contributions

Conceived and designed the experiments: Xia Hu, Jiamin Yu, Hui Chen, Chunyan Wang. Performed the experiments: Xia Hu, Jiamin Yu. Analyzed the data: Xia Hu, Jiamin Yu, Chunyan Wang. Contributed reagents/materials/analysis tools: Xia Hu. Wrote the paper: Xia Hu, Chunyan Wang.

Conflicts of Interest

The authors declare no conflict of interest.

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