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INVERTEBRATE MICROBIOLOGY

Nitrogen-Fixing and Uricolytic Bacteria Associated with the Gut of *Dendroctonus rhizophagus* and *Dendroctonus valens* (Curculionidae: Scolytinae)

Jesús Morales-Jiménez · Arturo Vera-Ponce de León · Aidé García-Domínguez · Esperanza Martínez-Romero · Gerardo Zúñiga · César Hernández-Rodríguez

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Abstract The bark beetles of the genus *Dendroctonus* feed on phloem that is a nitrogen-limited source. Nitrogen fixation and nitrogen recycling may compensate or alleviate such a limitation, and beetle-associated bacteria capable of such processes were identified. *Raoultella terrigena*, a diazotrophic bacteria present in the gut of *Dendroctonus rhizophagus* and *D. valens*, exhibited high acetylene reduction activity in vitro with different carbon sources, and its *nifH* and *nifD* genes were sequenced. Bacteria able to recycle uric acid were *Pseudomonas fluorescens* DVL3A that used it as carbon and nitrogen source, *Serratia proteomaculans* 2A CDF and *Rahnella aquatilis* 6-DR that used uric acid as sole nitrogen source. Also, this is the first report about the uric acid content in whole eggs, larvae, and adults (male and female) samples of the red turpentine beetle (*Dendroctonus valens*). Our results suggest that the gut bacteria of these bark beetles could contribute to insect N balance.

J. Morales-Jiménez · A. Vera-Ponce de León · A. García-Domínguez · C. Hernández-Rodríguez (✉)
Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prol. De Carpio y Plan de Ayala, Col. Sto. Tomas, Mexico, Distrito Federal 11340, Mexico
e-mail: chdez38@hotmail.com

E. Martínez-Romero
Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Av. Universidad S/N, 62210 Cuernavaca, Morelos, Mexico

G. Zúñiga
Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prol. De Carpio y Plan de Ayala, Col. Sto. Tomas, Mexico, Distrito Federal 11340, Mexico

Present Address:
J. Morales-Jiménez
Laboratorio de Ecología Molecular, Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana—Xochimilco, Distrito Federal 04960, Mexico

Introduction

Insects have a wide variety of diets and different nutritional sources, some rich in assimilable nitrogen sources, like blood, and others poor in these compounds, such as plant sap, phloem and wood. The dietary requirements can be complemented by microorganisms, which expand the biosynthetic or degradative capabilities of insects [19]. Microorganisms associated with insects feeding on phloem can supply essential amino acids, vitamins, fatty acids and sterols [3, 65]. Particularly, symbiotic interactions between microorganisms and bark beetles seem to be crucial to insect survival in nutritionally nitrogen poor substrate as bark and phloem. These insects need to elevate 16- to 26-fold the nitrogen contained in their diet [3, 28, 68]. For example, Ayres et al. [3] estimated that *Dendroctonus frontalis* had nitrogen-use efficiency (NUE) of 216 % to complete its metamorphosis from egg to adult if one adult contain around 105.7 μg of nitrogen with a consumption of 5.7 mg of phloem with 49 μg of nitrogen (0.86 %). Obviously, NUE values higher than 100 % are impossible. Thus, other potential nutritional strategies to increase the amount of assimilable nitrogen and NUE in *Dendroctonus* would be by association with nitrogen-fixing and uricolytic bacteria [20].

All nitrogen-fixing organisms are prokaryotes, which suggest that this metabolic capacity was developed early in evolution [54]. However, no eukaryotic organism is capable of fixing nitrogen. Fungi and animals require nitrogen bound in biomolecules to satisfy heterotrophically their nitrogen requirements but the fixed nitrogen available is a limiting nutrient in most of environments. To solve the limited access to nitrogen in some insects whose diets are

deficient, mutualistic interactions with diazotrophic bacteria may be established [42]. The association between nitrogen-fixing prokaryotes and insects was reported in termites [6, 9], and recently the expression of bacterial *nifH* genes, which encode the iron protein (dinitrogenase reductase) a key subunit of the nitrogenase complex [16], was detected in the bacterial microbiota associated with guts of lower and higher termites [30]. Likewise in bark beetles [11, 38], wood-eating cockroach [10], scarabaeid beetle [13], Mediterranean fruit fly [5] and stag beetle [34] nitrogen-fixing bacteria and their *nifH* genes and/or nitrogenase enzymatic activity have been reported. In the genus *Dendroctonus*, many bacteria previously reported as nitrogen-fixing microorganisms have been isolated (e.g., *Pantoea agglomerans*, *Rahnella aquatilis*, *Stenotrophomonas maltophilia*, and *Raoultella terrigena*) [7, 11, 38, 45, 71], but only *P. agglomerans* isolates have showed nitrogenase activity in vitro [11].

Uric acid is the main nitrogenous waste compound excreted by the majority of terrestrial insects [14, 40, 41]. *Periplaneta americana* harbors *Blattabacterium* sp., an intracellular bacterial symbiont that recycles ammonia and urea and excretes amino acids, but it is unable to assimilate uric acid. No uricase gene in *Blattabacterium* spp. genomes has been detected, suggesting that the first enzyme to degrade uric acid in *P. americana* could be encoded by the insect or other bacteria [59]. Several hindgut bacteria of *Reticulitermes flavipes* are able to convert uric acid to CO_2, NH_3 and acetate, and some evidence indicates that nitrogen from uric acid is incorporated in termite tissues [50–52]. Also *Erwinia*-like bacteria detected in the midgut of the shield bug *Parastrachia japonensis* produce a uricase that degrades uric acid and excrete amino acids to insect gut [32]. All evidence suggests that uricolytic bacteria play an important role in uric acid recycling and contribute to the more efficient use of nitrogen sources by insects that feed on poor-nitrogen diets.

Dendroctonus valens is a bark beetle widely distributed in North and Central America, where infest stumps and pine trees attacked by other bark beetles [69]. Recently, this species was introduced in China, where it is reported attack and kill its host trees (*Pinus tabuliformis*) [37, 70]. *Dendroctonus rhizophagus* is an endemic species of the Northwest of Mexico, where infests and kills seedlings and young saplings <3 m height of 11 pine species [36, 60]. Bark beetles of genus *Dendroctonus* are phloeophagous insects that need to use the nitrogen contained in their food with a high effectiveness, therefore it is possible that fungus, yeasts and bacteria are responsible of the phloem nutritional improvements with essential amino acids, vitamins, fatty acids and sterols [11, 56, 65]. The presence of mycangial fungi seems to have a considerable impact on the resource-use efficiency of bark beetles with mycangium, and their absence result in higher phloem consumption. Therefore, in bark beetles without mycangium different nitrogen acquisition strategies could be crucial to their survival [3, 65]. To know if bark beetles gut-associated bacteria can achieve this function, we tested the nitrogen-fixing ability of *R. terrigena* isolated from the gut of *D. rhizophagus* in a previous study [39]. In addition, we assayed and quantified the uricolytic activity of gut-associated bacteria previously isolated from *D. rhizophagus* and *D. valens* [38, 39]. Lastly, to have an overview on the importance of uricolytic activities in *Dendroctonus* spp., the density of uricolytic bacteria was determined and the uric acid content in whole insects of different life stages was evaluated.

Material and Methods

Beetle Collection

Adult females and males, larvae and eggs of *D. rhizophagus* and *D. valens* were collected from two geographical locations in Mexico (Table 1). Samples were obtained directly from galleries of infested pine trees using fine forceps during the different stages of their life cycle. Live adults, larvae and eggs were transported to the laboratory in sterile vials containing sterile moist paper and disinfected superficially with 70 % ethanol and submerged repeatedly in phosphate buffer solution (PBS) to avoid external contamination. Adult insects were dissected under sterile conditions, and gut extraction was performed by the elimination of elytra, wings, and tergites to expose the insect's abdomen A longitudinal incision was made on larvae to expose the gut. Guts were transferred individually to 1.5-ml microcentrifuge tubes with 0.2 ml of nutrient broth (beef extract 3 $g l^{-1}$ and peptone 5 $g l^{-1}$) (Difco).

Nitrogen-Fixing Bacteria from *Dendroctonus* spp.

Acetylene Reduction Assay

The nitrogen fixation capability of *R. terrigena* DR-E5 (JN712166) previously isolated from *D. rhizophagus* and *D. valens* (data not shown) was evaluated using the acetylene reduction assay (ARA) in a nitrogen-free semisolid medium (Haahtela medium) [23] with the following carbon sources: glucose, fructose, lactose, sucrose, cellobiose, raffinose, xylose, mannitol, malic acid and acetate. Moreover, nitrogen fixation was evaluated in a modified medium containing four different carbon sources (glucose, sucrose, malic acid and mannitol) (WATC4 medium) [7]. ARA was performed in triplicate using closed vessels (10 ml) containing 5 ml semisolid culture media inoculated with a single colony isolated from nitrogen-free solid medium. The

Table 1 *Dendroctonus* spp. samples used in this study

Location in Mexico	Latitude/ Longitude	Host tree	Insect samples			
			Insect species	Life cycle stage	Number of specimens collected	Collection date
San Juanito, Bocoyna, Chihuahua	27°92′N/107°60′W	*Pinus engelmannii*	*D. rhizophagus*	Larvae	15	January 2010
				Adults	15	May 2009
Los Pozos, Gómez Farias, Jalisco	19°88′N/103°40′W	*Pinus montezumae* *Pinus leiophylla*	*D. valens*	Eggs	500	April 2009
				Larvae	40	April 2009
				Adults	50	April 2009

media were incubated for 24 h at 28 °C, and acetylene-enriched atmosphere was injected to achieve 10 % final concentration. Closed vessels without inoculated bacteria were used as negative controls, and vials inoculated with *Klebsiella variicola* 6A3 were used as positive controls. The ethylene formed by nitrogenase activity was measured with a Varian 3300 (Walnut Creek, CA, USA) gas chromatograph fitted with a hydrogen flame ionization detector and a 3.2 mm×2 m stainless column packed with Poropak N (80–100 mesh). The column was operated isothermally at 95 °C with nitrogen as carrier gas at a flow rate of 30 ml min^{-1} and detector temperature was set at 105 °C. The ethylene production was estimated as the integration of the curve area and the values were transformed to nmol of ethylene h^{-1} [25]. Finally, paired Student's *t*-test was used to identify significant differences between the ARA activity of positive control (*Klebsiella variicola* 6A3) and *R. terrigena* DR-E5 with each carbohydrate tested.

Phylogeny of nifH and nifD Genes from Raoultella terrigena DR-E5

To know the phylogenetic relationships of the nitrogen fixation genes from *R. terrigena*, total DNA was extracted from isolated colonies according to Hoffman and Winston [29]. The *nifH* and *nifD* genes were amplified using a set of primers described previously [43]. Final concentrations for 25-μl PCR reactions were as follows: 10 ng of total DNA isolated from each colony, 0.8 pM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 U of *Taq* polymerase, and 1× Taq polymerase buffer (Invitrogen Life Technologies, Sao Paulo, Brazil). The reaction conditions to amplify *nifH* were: 94 °C for 7 min; 35 cycles of 60 s at 94 °C, 60 s at 52 °C, and 60 s at 72 °C and a final extension at 72 °C for 7 min, and to amplify *nifD* were: 94 °C for 7 min; 35 cycles of 60 s at 94 °C, 60 s at 60 °C, and 60 s at 72 °C; and a final extension at 72 °C for 7 min. The products were separated in a 1 % (w/v) agarose gel in TAE. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced in an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) using the same primers. The sequences of *nifD* and *nifH* genes were deposited in the GenBank database under the accession numbers JX080197 and JX080198, respectively. Amino acid deduced sequences of *nifH* and *nifD* genes were aligned with sequences obtained from the GenBank database using CLUSTAL X [66] and edited and confirmed visually in BIOEDIT [24]. PROTTEST was used to select the best-fit model of protein evolution [1]. The best model according to Akaike information criterion was LG + G for both *nifH* and *nifD* genes (α=0.494 and 0.912 for the gamma distribution for *nifH* and *nifD* genes, respectively) [48]. Maximum likelihood analyses were performed using PhyML ([22]; http://atgc.lirmm.fr/phyml/) and the confidence at each node was assessed by bootstrap after 1,000 pseudoreplicates [27]. *nifH* and *nifD* genes from *Rhodopsuedomonas palustris* were used as outgroup to each phylogeny.

Uricolytic Bacteria from *Dendroctonus* spp.

Isolation of Uricolytic Bacteria

Individual guts from adult insects from *D. rhizophagus* and *D. valens* collected for this study, were placed separately in sterile microcentrifuge tubes and crushed in 200 μl of nutrient broth (Difco). Serial 10-fold dilutions from 10^{-1} to 10^{-6} were spread on duplicate plates of nutrient agar (Difco) with 0.15 % uric acid. Plates were incubated at 28 °C for 2 to 5 days; each colony with a clear halo suggestive of uric acid utilization was categorized, according to its colonial morphology and microscopic appearance, and counted. All isolates were stored at −70 °C.

16S rRNA Gene Analysis

To identify new uricolytic bacteria isolates the 16S rRNA gene sequence was used. DNA extraction and PCR of

uricolytic bacteria were performed following the protocols previously reported [38]. 16S rRNA gene was amplified using primers 8 forward (5′-GCGGATCCGCGGCCGCTG CAGAGTTTGATCCTGGCTCAG-3′) and 1,492 reverse (5′-GGCTCGAGCGGCCGCCCGGGTTACCTTGTTACG ACTT-3′) [55]. The amplification reaction was performed as above with a denaturation step of 7 min at 94 °C followed by 35 cycles at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s, and a final elongation step of 72 °C for 10 min. The bacteria identification inferred by phylogenetic analysis was realized by Maximum likelihood with sequences of 16S rRNA genes. All isolated bacteria sequences were compared with the non-redundant GenBank library using BLAST search [2]. A collection of taxonomically related sequences were obtained from the National Center for Biotechnology Information Taxonomy Homepage (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/). DNA sequences were aligned using CLUSTAL X [66] and edited and confirmed visually in BIOEDIT [24]. Maximum likelihood analyses were performed using PhyML ([22]; http://atgc.lirmm.fr/phyml/). MODELTEST 3.06 [47] was used to select appropriate models of sequence evolution by the AIC model [48]. The GTR+G model (α=0.196 for the gamma distribution; A=0.25, C=0.22, G=0.31, T=0.20) was selected for the tree search. The confidence at each node was assessed by bootstrap after 1,000 pseudoreplicates [27]. *Anabaena affinis* was used as the outgroup. The similarity percentages among sequences were calculated using MatGAT v. 2.01 software [12]. The limits for genus and species were set at 95 % and 97 %, respectively [62]. The 16S rRNA gene sequence was deposited in the GenBank database under the accession number JX080199.

Uricolytic Activity

The uricolytic activity of 26 bacterial isolates obtained in previous studies of gut-bacterial communities of *D. valens* and *D. rhizophagus* [38, 39] and ten new isolates were tested. Enzyme activity was indexed as the diameter of the colony plus the clear zone divided by the diameter of the colony. The halo diameters of two identical colonies were measured. Some of the isolates with the highest index of uricolytic activity were used to assess bacterial growth with uric acid as sole nitrogen source, or as sole carbon and nitrogen sources. Microbial growth and uric acid utilization kinetics were performed using methods and media reported by Rouf and Lomprey [58]. The uric acid content was measured at 295 nm.

Determination of Uric Acid from Insects

Adult male or female, larvae or a pool of 100 eggs from *D. rhizophagus* and *D. valens* collected for this study during 2009 were manually crushed, homogenized with a plastic pestle into sterile microcentrifuge tubes with 500 µl of 1 M HCl, shaken vigorously on a Vortex, incubated at 60 °C for 15 min to denature proteins and centrifuged at 10,000×g for 10 min. The supernatant was neutralized with 500 µl of 1 M NaOH and centrifuged at 10,000×g for 10 min. An aliquot of the extract was filtered through a membrane of 0.45 µm porosity and subsequently degassed.

A standard stock solution of 500 µgml^{-1} uric acid (pH 10.3) was used to prepare a standard curve ranging from 0.5 to 100 µgml^{-1}. High-pressure liquid chromatography (HPLC) was used to quantify uric acid in the insects. The HPLC separation was performed with Varian 920-LC Analytical HPLC system (Walnut Creek, CA, USA) using a reverse-phase C18 Econosil column (250×4.6 mm i.d., 5 µm particle size) from Alltech Associates. The mobile phase was 95:5 water–methanol solution with 0.560 g of KH_2PO_4 and 0.480 g of Na_2HPO_4 per litter to adjust the pH to 6.6–6.7. One ampule of tetrabutylammonium phosphate monobasic solution (Sigma-Aldrich) as ion pair was added to each liter of mobile phase. The solution was filtered through a 0.45 µm membrane and degassed before use. The UV detector, set at 280 nm, was used for the quantification of uric acid. The analyses were performed isocratically at a flow rate of 1.0 ml/min and at room temperature. A total of 20 µl of each sample or standard was injected to HPLC. A Levene's test was used to determinate homogeneity of variances. Due to the variances were heterogeneous, the means of the uric acid content among sexes and larvae were compared using Welch ANOVA. Games–Howell post-hoc test were applied to establish pairwise comparisons among males, females and larvae. All statistical analyses were performed using SPSS statistical software (SPSS version 13.0 for Windows, SPSS Inc., Chicago, IL).

Results

Nitrogen-Fixing Bacteria

Raoultella terrigena DR-E5 showed nitrogenase activity in WATC4 and Haahtela media and harbored *nifD* and *nifH* genes. Nitrogen fixation activity of this bacterium was supported by all carbohydrates tested less acetate in Haahtela medium (Fig. 1). *R. terrigena* DR-E5 showed similar acetylene reduction rates to *K. variicola* 6A3 (positive control) when sucrose, malic acid and cellobiose were used as carbon and energy sources according to paired Student's t-tests (P>0.05, Table 2). Moreover, when fructose, glucose, lactose, mannitol, xylose and raffinose were used to support nitrogen fixation, *K. variicola* 6A3 exhibited higher acetylene reduction rates than *R. terrigena* DR-E5 (Student's t-tests, P<0.05). On the other hand, the highest

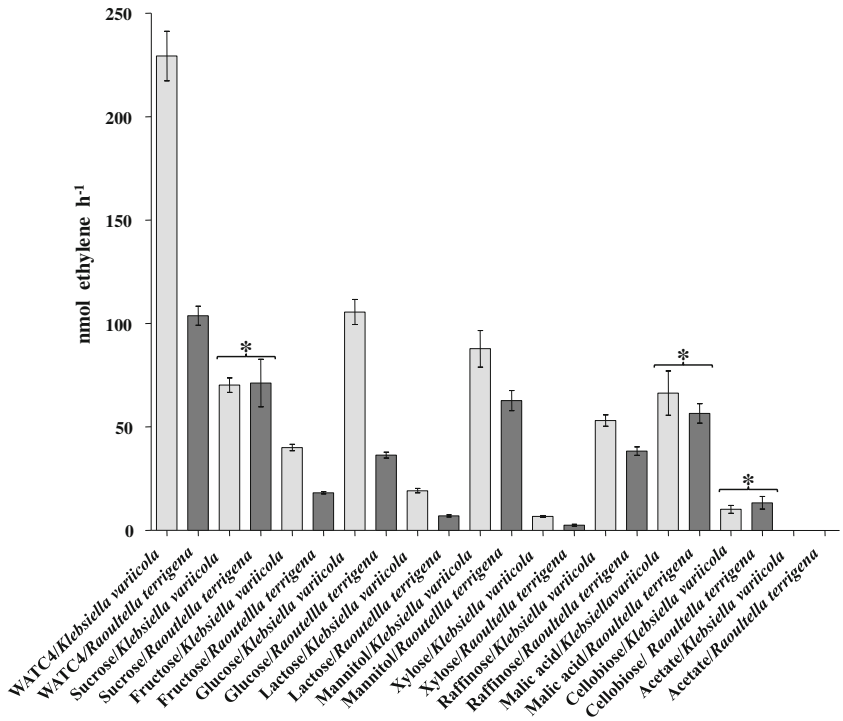

Fig. 1 Acetylene reduction assay of pure cultures of *Raoultella terrigena* DR-E5 isolated from *Dendroctonus rhizophagus* gut. *Klebsiella variicola* 6A3 was used as positive control. Bars represent standard errors. *Show the carbon sources without significant difference between the positive control (*K. variicola* 6A3) and *R. terrigena* DR-E5 (Student's *t*-tests, $P>0.05$)

nitrogenase activity of *R. terrigena* DR-E5 was detected in WATC4 media with 107.8±4.6 nmol ethylene h^{-1}.

Both nucleotide and deduced amino acid sequences of *nifH* and *nifD* genes of *R. terrigena* DR-E5 clustered with those from *K. pneumoniae*, *K. variicola* and *Pantoea* sp. At-9b (Figs. 2 and 3). Partial *nifH* gene nucleotide sequences exhibited from 88 to 91 % identity with the homologous genes of *Klebsiella* spp. and 87 % with *Pantoea* sp. The *nifD* gene sequence of *R. terrigena* DR-E5 showed high identities (82 %) with *Klebsiella* spp. and *Pantoea* sp. High identities of *NifH* (97–98 %) and *NifD* (96 %) proteins from *R. terrigena* DR-E5 were found with orthologous proteins of *Klebsiella* spp. and *Pantoea* sp., respectively.

Uricolytic Bacteria

The populations of uric acid degrading-bacteria in the gut of larvae, pupae, and adults of *D. valens* were $4.64\times10^4\pm 2.32\times10^4$, $3.67\times10^2\pm1.5\times10^2$ and $3.2\times10^4\pm1.64\times10^4$ CFU per gut, respectively. Some bacterial isolates from *D. rhizophagus* gut also showed in vitro uricolytic activity, but the plate count could not be determined. All colonies counted showed uricolytic activity in nutrient agar supplemented with uric acid at 0.5 %. *Pseudomonas fluorescens* DVL3A isolated and indentified from *D. valens* gut in this work (Fig. 4), *Serratia proteomaculans* 2A CDF and *R. aquatilis* 6-DR isolated in previous studies [38, 39] showed the highest activity in solid media with uric acid among all

Table 2 Nitrogenase activities by *R. terrigena* DR-E5 with different carbon sources

Carbon source	Nitrogenase activity[a]	*P*
Glucose, sucrose, malic acid and mannitol (WATC4)	103.8±4.6	5.9×10^{-3}
Sucrose	71.22±11.5*	4.6×10^{-1}
Fructose	18.15±0.54	4.2×10^{-3}
Glucose	36.38±1.45	4.9×10^{-3}
Lactose	6.46±0.44	1.3×10^{-3}
Mannitol	62.72±4.9	1.2×10^{-2}
Xylose	2.5±0.39	8×10^{-4}
Raffinose	38.32±2	3.2×10^{-3}
Malic acid	56.55±4.73*	2.7×10^{-1}
Cellobiose	13.27±3.06*	6.4×10^{-2}
Acetate	0±0*	1.1×10^{-1}

[a]Values are means ± standard error of triplicate experiments. Activities are expressed in nmol of ethylene h^{-1}

*Values are not statistically different in comparison with the positive control (*Klebsiella variicola* 6A3) using the same carbon source (Student's *t*-tests, $P>0.05$)

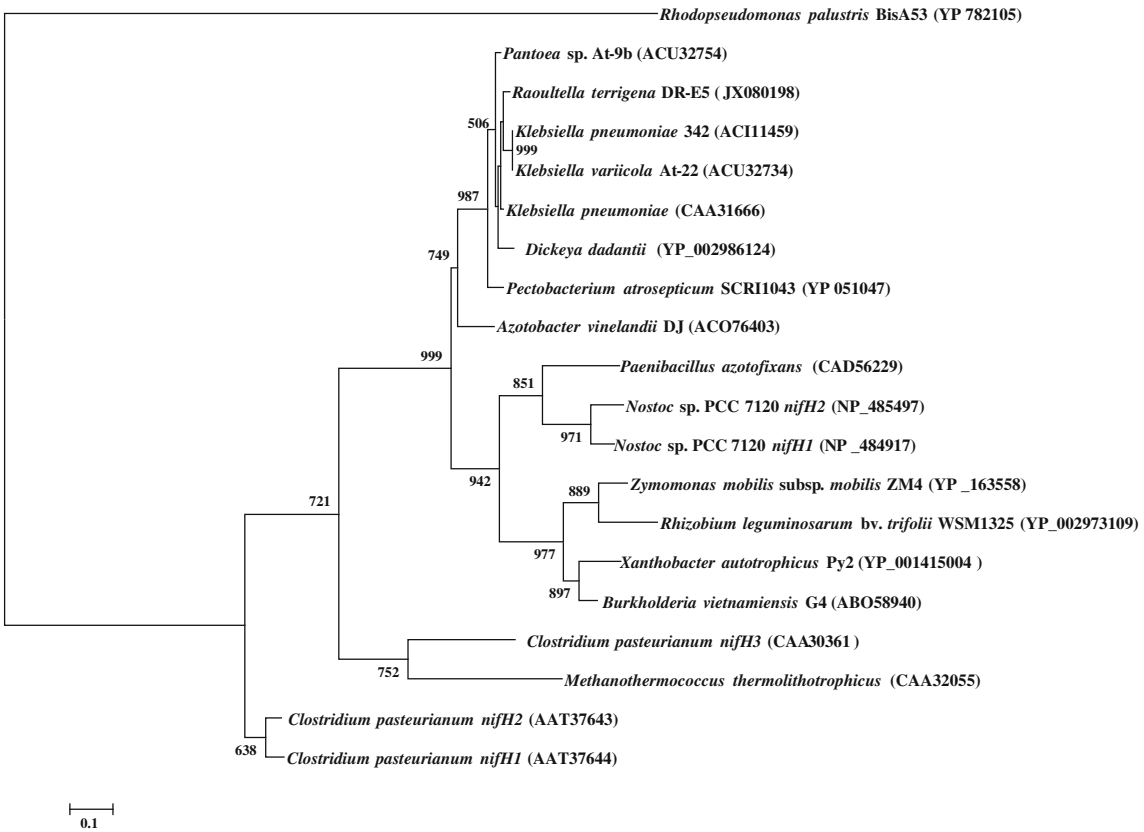

Fig. 2 Maximum likelihood tree (−ln L=3,253.35) of amino acid sequences deduced from *nifH* of *Raoultella terrigena* DR-E5 and other sequences from the database. The *nifH* amino acid sequence of *Rhodopseudomonas palustris* was used as outgroup. The LG + G model (a=0.49 for the gamma distribution) was selected for the tree search. Scale bar indicates 1 % estimated sequence divergence. Bootstrap support values ≥50 % are indicated on nodes

bacteria associated with *D. valens* and *D. rhizophagus* guts. All isolates grew well in mineral medium supplemented with uric acid and glucose; however the growth was slight to poor when glucose was omitted from the medium. All strains grew aerobically with uric acid as sole nitrogen source in mineral medium and took 5 and 12.5 h to remove completely the uric acid contained in the medium (Fig. 5). Notably, *P. fluorescences* DVL3A was the only strain capable of using uric acid as sole nitrogen, carbon and energy source and depleted the substrate in only 8 h (Figs. 5 and 6).

Uric Acid Content in Adult Male and Female, Larvae and Eggs of *D. valens*

The content of uric acid per individual larvae, female and male adults of *D. valens* was 0.79±0.07, 5.5±0.82, and 3.3±1.03 μg, respectively (Fig. 7). Particularly, the lowest content of uric acid per individual was detected in larvae with 0.44–1.32 μg. The level increased in adult males and females varied from 1.28–9.31 and 2.09–9.2 μg, respectively. Levene's test revealed that homogeneity of variance assumption was no met (P=0.0008). As such, the Welch-ANOVA was used. A statistically significant difference was observed among the uric acid content of larvae, males and females in Welch-ANOVA ($F_{2,\ 8.095}$=17.83, P<0.001, est. ω^2=0.335). An alpha level of 0.5 was used for the subsequent analysis. Pos-hoc comparisons using Games–Howell tests showed that the uric acid content between females and larvae was statistically different (P=0.003); the mean difference was 4.73 and the effect sizes was 1.56. Meanwhile, the uric acid content of males was not statistically different than the content of females and larvae (P=0.26 and P=0.11, respectively). Lastly, the uric acid content of individual egg was 19.53±3.97 ng.

Discussion

Our results show that the nitrogen-fixing bacterium *R. terrigena* DR-E5 could use several carbon sources to support nitrogenase activity. *NifH* and *nifD* genes were closely related with the orthologous genes of *Klebsiella* spp. In addition we reported the presence of uricolytic bacteria in the gut of *D. rhizophagus* and *D. valens* and their capability to use uric acid as a sole nitrogen and/or carbon source in vitro. Furthermore, the content of uric acid was estimated in

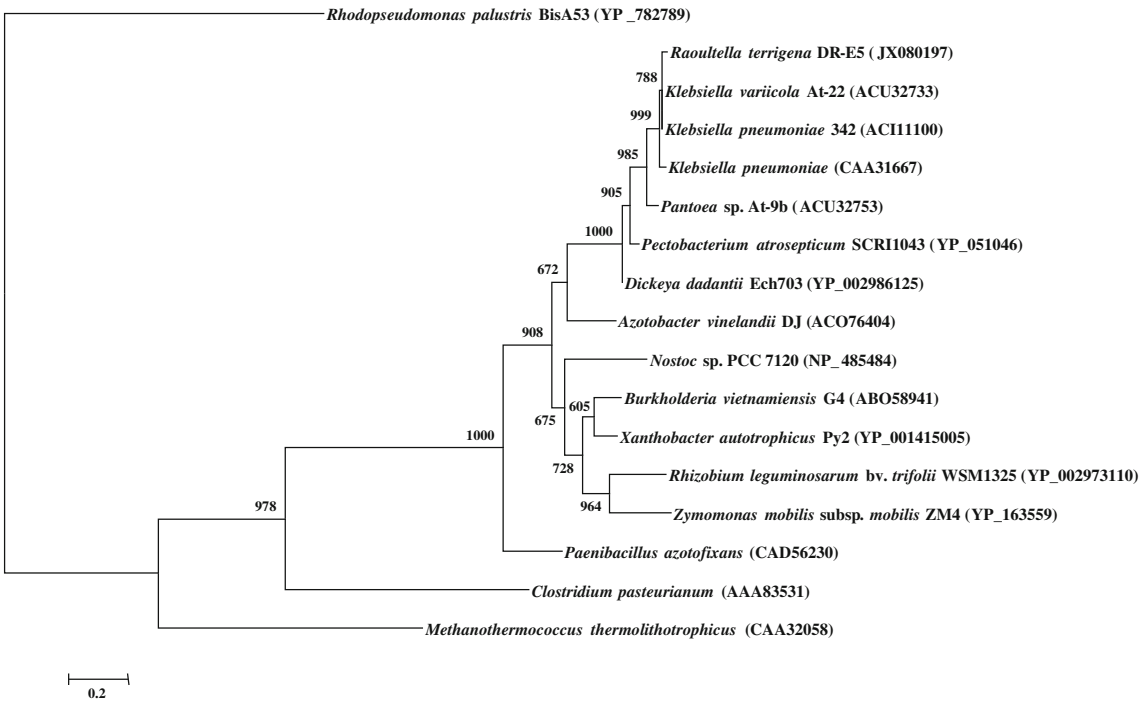

Fig. 3 Maximum likelihood tree (−ln *L*=6,176.32) of amino acid sequences deduced from *nifD* of *Raoultella terrigena* DR-E5 and other sequences from the database. The *nifD* amino acid sequence of *Rhodopseudomonas palustris* was used as outgroup. The LG + G model (*a*=0.912 for the gamma distribution) was selected for the tree search. Scale bar indicates 20 % estimated sequence divergence. Bootstrap support values ≥50 % are indicated on nodes

adult males and females, larvae and eggs of *D. valens*. These findings suggest that nitrogen fixation and uric acid recycling can be performed by specific bacterial guilds in the bark beetles gut. Both processes potentially could enhance the nitrogen available for bark beetles introducing assimilable nitrogen in the gut and contributing to NUE.

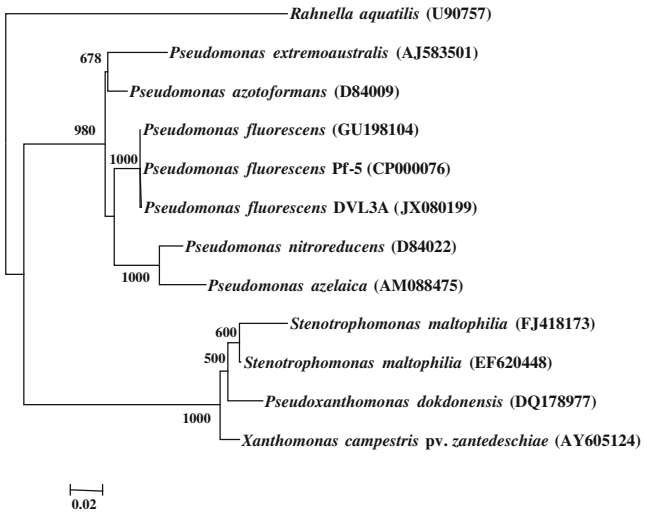

Fig. 4 Maximum likelihood tree (−ln *L*=−4,711.03006) of uricolytic bacterium isolated in this study from *Dendroctonus valens* gut. The 16S rRNA sequence of *Rahnella aquatilis* was used as outgroup. Scale bar indicates 2 % estimated sequence divergence. Bootstrap support values ≥50 % are indicated on nodes

The content of nitrogen in phloem of *Pinus taeda* L. was of 0.86±0.03 % of dry mass; meanwhile, the nitrogen content of adult insects was 11.56±0.13 % of dry mass [3]. Under this scenario, beetles need to concentrate around 13.44 times the nitrogen present in pine phloem. In other insects with nitrogen-poor diets, nitrogen-fixing bacteria and uricolytic bacteria play an important role in nitrogen intakes to their host [5, 9, 10, 32, 49–53, 59]. Perhaps these metabolic capabilities allow bark beetles to colonize the subcortical tissues and use a substrate like phloem in which nitrogen sources are spatially and temporally variable and extremely limited.

Members of the Enterobacteriaceae family have been consistently associated with the gut of several insects and their capability to fix nitrogen has been broadly demonstrated [11, 46, 49, 53]. *R. terrigena* DR-E5 harbored *nifH* and *nifD* genes, and exhibited nitrogenase activity in vitro. Phylogenetic relationships of *nifH* gene and its deduced protein of this bacterium clustered with molybdenum containing nitrogenases found in other members of Enterobacteriaceae associated with insect guts or other environments [16, 26, 44, 46, 57].

Moreover, *R. terrigena* was able of utilize different substrates for nitrogen fixation, including sucrose, fructose, glucose, xylose and cellobiose, all these carbohydrates are present in the phloem [18, 21, 63]. The highest nitrogen activity was detected in a medium with four carbon sources

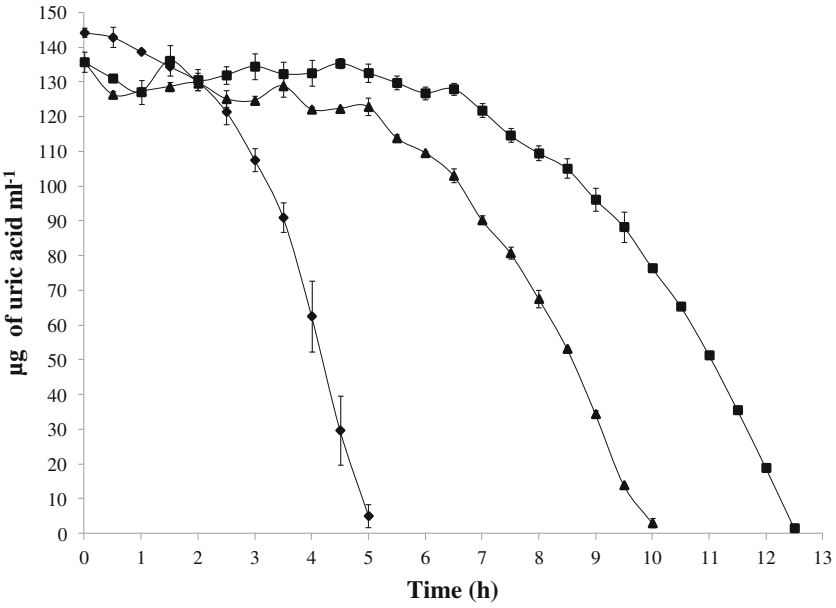

Fig. 5 Uric acid degradation with glucose as carbon source by bacteria isolated from *Dendroctonus* spp. gut. *Filled diamond*, *Pseudomonas fluorescens* DVL3A; *filled square*: *Serratia proteamaculans* 2A CDF; *filled triangle*: *Rahnella aquatilis* 6-DR. Values are expressed as means ± SE of three independent experiments

followed by activity with sucrose, mannitol and malic acid. Similarly, some isolates of *P. agglomerans* from the root of *Phalaris arundinacea* and *Phelum pratense* showed high nitrogenase activities when mannitol and sucrose as were tested as sole carbon sources [23]. With xylose as sole carbon source, these strains reduced acetylene to ethylene at elevated rates [23]; however *R. terrigena* DR-E5 from *D. rhizophagus* had a poor activity. Acetylene reduction activity was higher with *R. terrigena* DR-E5 than *Pantoea* spp. isolated from fungus gardens of leaf-cutter ants, but lower than *K. variicola* used as control (*K. variicola* 6A3) and the isolates from fungus gardens [46]. *R. terrigena* DR-E5 had a low nitrogenase activity using cellobiose as a sole carbon source; this substrate could be produced in the beetle gut as a product of cellulose degradation or in the pine galleries and feeding chamber of these insects. It seems that other different carbon sources used by the bacteria may sustain nitrogen fixation in the bark beetles gut.

The microbial capacity to metabolize uric acid has long been known [32, 50–52, 58, 59]. According to the uricolytic bacteria populations recorded in this study and the total heterotrophic populations reported previously [38], this microbial group represent around 1.65 % of the total heterotrophic culturable bacteria associated with the adult insect gut of *D. valens*. This abundance is similar to populations of this bacterial group previously reported in *Reticulotermes*

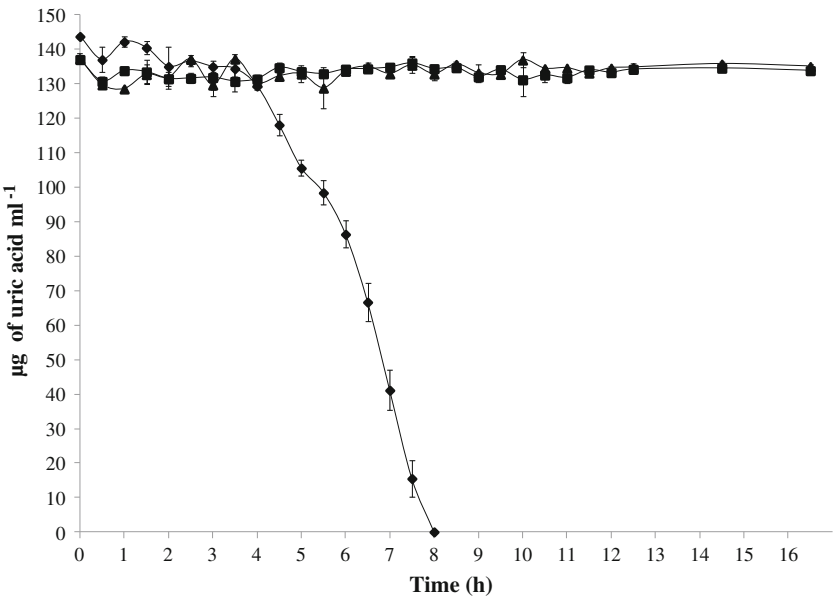

Fig. 6 Uric acid degradation by bacteria isolated from *Dendroctonus* spp. gut. Uric acid was the sole carbon, nitrogen and energy source. *Filled diamond*: *Pseudomonas fluorescens* DVL3A; *filled square*: *Serratia proteamaculans* 2A CDF; *filled triangle*: *Rahnella aquatilis* 6-DR. Values are expressed as means ± SE of three independent experiments. Uric acid was the sole carbon, nitrogen and energy source

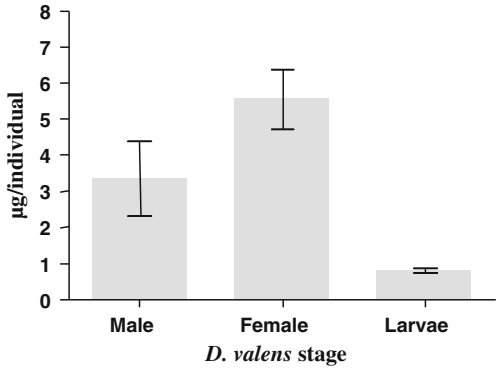

Fig. 7 Uric acid contents in male and female adults and larvae of *D. valens*. Values are expressed as means ± SE (males, n=7; females, n=7; larvae, n=30)

flavipes [50]. Possibly the uric acid is released from the Malpighian tubules to the *Dendroctonus* gut, as was demonstrated in *R. flavipes* [52] and its recycling process could be similar. *P. fluorescens* DVL3A, *Serratia proteomaculans* 2A CDF, and *R. aquatilis* 6-DR were previously detected in the gut microbiota of *D. frontalis*, *D. rhizophagus* and *D. valens*, but their uricolytic capacity was not evaluated [38, 39, 67]. All isolates were able to use aerobically uric acid as sole nitrogen source in a liquid medium, but *P. fluorescens* DVL3A could utilize the uric acid as the only source of carbon, nitrogen and energy. The capability to use uric acid as carbon source, or carbon, nitrogen and energy sources by *Pseudomonas* and *Serratia* species has long been known [4, 58]. A strain of *P. fluorescens* isolated from waste materials of chicken was able to use uric acid and glucose as carbon sources, but no growth was observed only with uric acid as a sole source of carbon. However, a *P. aeruginosa* strain isolated from the same environment was able to use uric acid as a sole source of carbon, nitrogen and energy [58]. Meanwhile, to our knowledge, this is the first report of *Rahnella* strains as uricolytic bacteria.

The participation of gut bacteria in uric acid recycling has been broadly documented in several insects like *R. flavipes*, *P. americana*, *P. japonensis* [14, 15, 32, 52]. The possible role of gut uricolytic bacteria associated with *Dendroctonus* species could be similar to the cases of termites and cockroaches feeding on a nitrogen-poor diet. In those insects, the symbiotic bacteria seem to play a central role in making the nitrogen contained in uric acid available to the insect [14, 15, 32, 52].

The uric acid contained in eggs, larvae, adults of *D. valens* seems to be low compared with the concentration reported in the shield bug *P. japonensis*. While the adults of red turpentine beetle contain only 1.2–9.3 µg, the adults of the hemipteran insect have 30–50 µg in the early reproductive stage of both sexes and this amount increase to 200 µg after oviposition [31]. However, similar concentrations to those obtained with adults and eggs of *D. valens* have been observed in the brown planthopper (*Nilaparvata lugens*) whose content of uric acid was around 11.76 µg in nymphs and 0.13 µg in eggs [30]. In *N. lugens*, in particular, yeast-like endosymbionts seem to be the ones responsible for nitrogen recycling. The symbionts' uricase initiates uric acid reutilization when the insects encounter to a nitrogen deficiency, and it is vertically transmitted to the offspring [30, 61]. A similar case could be occurring in insects of the genus *Dendroctonus*. However, little is known about the possible functional roles of yeast associated with bark beetles. In the literature, the beetle-associated yeast has been involved in pheromone production, substrates digestion and detoxification of insect harmful chemicals [8, 17, 35, 56]. The participation of yeast in the insect nitrogen balance must be demonstrated. On the other hand, the concentration of uric acid present in *D. valens* larvae was lower than the uric acid contained in adults of both sexes. A similar tendency was observed in *P. japonensis* where the uric acid increased during ovarian development and after the oviposition [32]. This is the first study to quantify the uric acid content of an entire bark beetle species. However, further studies must be conducted to determine the uric acid content in other stages of the life cycle of *Dendroctonus* (e.g., in each larval instar, pupae, adult emerging and attacking, mating, oviposition, old adults), and estimate the relevance of this substrate on bacterial recycling of nitrogen.

In brief, our results show that uric acid recycling and nitrogen fixation by gut bacteria is possible and perhaps very important to the nitrogen budgets of bark beetles of the genus *Dendroctonus*. Both processes could elevate the available nitrogen or the NUE. Nitrogen fixation and uricolysis share the same final product: the NH_3 [33, 50, 51]. This compound could be assimilated directly by the insects via glutamine synthetase activity. However, this scenario could be difficult with nitrogen fixation due to it is one of the most metabolically expensive processes in biology [64]. Alternatively, in uricolysis and nitrogen fixation the ammonia could be first incorporated into bacterial biomass, to become available later by death and lysis of microbial cells in the gut or gallery environments. Also, amino acids could be released by diazotrophs and uricolytic bacteria into the gut of their insect host. These bacterial guilds, as a consequence of bark beetles of the genus *Dendroctonus* that feed on a poor nitrogen source like the phloem of conifer trees [3], seem to be fundamental for bark beetles to have a high NUE.

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