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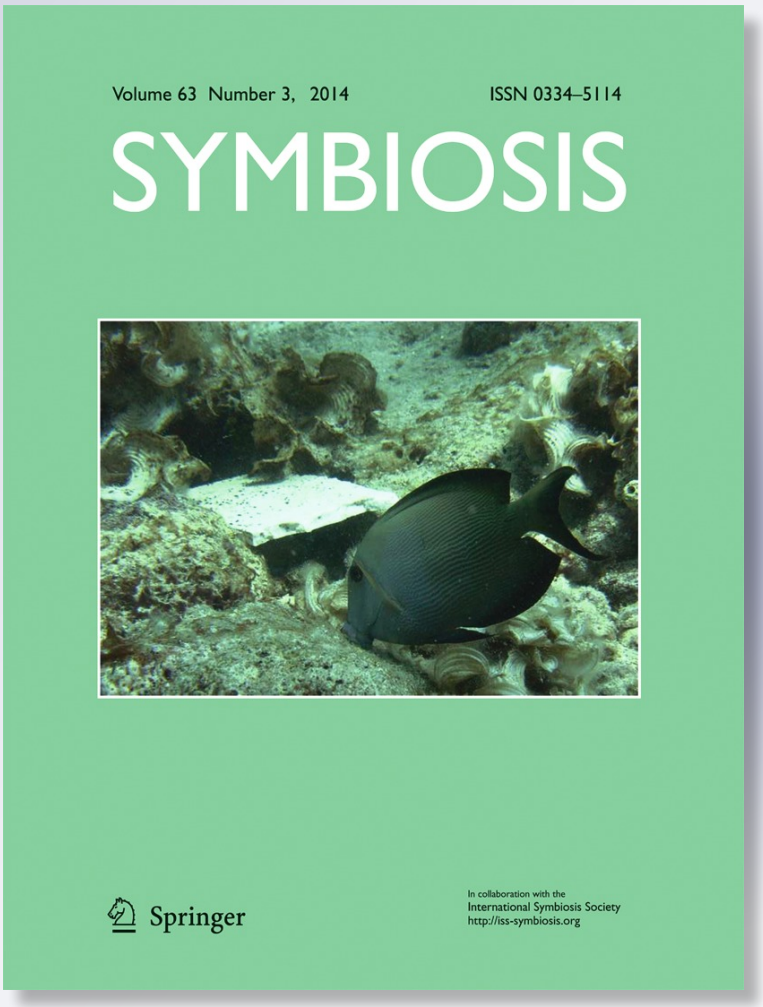

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Uric acid-degrading bacteria in the gut of the invading apple snail *Pomacea canaliculata* and their possible symbiotic significance

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Abstract In previous studies we have mapped uric acid deposits in specialized tissues of the invading apple snail *Pomacea canaliculata*. Uric acid stores function as reservoirs of combined nitrogen in insects. The first step for recycling uric acid nitrogen involves the action of uricase and here we explored the occurrence of uricase-positive bacteria in the gut of this snail. Six bacterial strains assigned to the genera *Pseudomonas*, *Enterobacter*, *Citrobacter* and *Lactococcus* were isolated on the basis of their ability to grow in vitro in a medium containing uric acid as the only carbon and nitrogen source. Uricase specific activity could be determined in three of these genera (not in *Citrobacter*), showing optimal pH values ranging 7.3–8.7 and optimal substrate concentrations (ranging 25–30 μM). These uricolytic bacteria may participate in recycling of combined nitrogen in this snail.

Keywords Nitrogen recycling · Gut uricolysis · Apple-snail · Urate stores

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1 Introduction

Uric acid was discovered by von Sheele in 1776 in human urine and bladder stones and was considered an anomaly, frequently associated with human gout (Roberts 1890; Nuki and Simkin 2006). However, it was later recognized that uric acid was physiologically indispensable for nitrogen excretion by many animals (Roberts 1890). Also, other physiological roles of uric acid were proposed later, namely: as an antioxidant (Becker 1993) and as a reservoir of combined carbon and nitrogen which may be recycled into proteins (Nolfi 1970).

Even later, it was shown that uricolytic bacteria participate in recycling of combined nitrogen from uric acid stores, particularly in the fat body of termites, cockroaches and other insects (e.g., Brune and Ohkuma 2011; Costa-Leonardo et al. 2013; Potrikus and Breznak 1981; Warnecke et al. 2007; Tokuda et al. 2008; Cochran 1985; Wren and Cochran 1987; Kashima et al. 2006; Sasaki et al. 1996). Uricolysis may occur within the fat body itself (Patiño-Navarrete et al. 2014) or after uric acid transfer into the gut lumen via nephridia (Potrikus and Breznak 1981).

Uric acid is stored as intracellular crystalloids in specialized tissues of the gastropod *Pomacea canaliculata* Lamarck 1822 (Caenogastropoda, Architaenioglossa, Ampullariidae) (Vega et al. 2007; Giraud-Billoud et al. 2008). Uric acid released from these stores has been shown to protect this snail against the damaging effects of tissue reoxygenation following estivation (Giraud-Billoud et al. 2011, 2013). However, these urate stores may also be used as reservoirs of combined nitrogen, as it occurs in insects (e.g., Potrikus and Breznak 1981; Patiño-Navarrete et al. 2014), though this possibility has not been explored. Such recycling requires the conversion of uric acid into simpler molecules, and the first step in this conversion is oxidation to allantoin, a reaction that may occur non-enzymatically (as it has been shown in *P. canaliculata*; Giraud-Billoud et al. 2011; Giraud-Billoud et al. 2013) or that

it may be catalyzed by uricase (EC 1.7.3.3, factor-independent urate hydroxylase),, which is found in some tissues of this snail (Giraud-Billoud et al. 2011). However, urate-storing tissues in *P. canaliculata* do not contain 'mycetocytes', i.e., the bacteria-containing cells in the fat body of insects (Costa-Leonardo et al. 2013). Hence, and according to previous reports (e.g., Potrikus and Breznak 1981) the gut became a potentially significant site to explore the occurrence of uricolytic bacteria. To our knowledge, this is the first report of uric acid-degrading bacteria in the gut of a gastropod.

2 Material and methods

Samples of the bacterial biota were obtained from 7 mature female *Pomacea canaliculata* (~6 months old) from a cultured strain. The strain's original stock was collected between 1993 and 1996 (several collections) at the Rosedal Lake (34°34' S; 58°25' W, Palermo, Buenos Aires, Argentina) and it has been maintained in our laboratory ever since. Specimens of the original population and the cultured strain were deposited at the collection of Museo Argentino de Ciencias Naturales (Buenos Aires, Argentina lots MACN-In 35707 and MACN-In 36046, respectively). Animals were maintained in controlled conditions of photoperiod (14 h light/day) and temperature (23–26 °C) which approximate spring natural conditions in central Argentina. Eight to ten snails were kept per 56 × 38 × 20 cm aquarium, where water level was kept ~4 cm deep, and was changed thrice weekly. The animals were fed *ad libitum* with lettuce from Mondays to Fridays, and also received high protein (40 %) fish food pellets (Peishe Car Shulet®, Argentina) as an *ad libitum* protein supplement on Thursdays. On Fridays the snails were also provided with excess toilet paper (Higienol Export®, Argentina) on Fridays. The latter provided cellulose as a nutrient and did not spoil the water during weekends, as excess lettuce or pellets would do.

The shell was wiped with 70 % ethanol and then cracked, while carefully avoiding contact of the outer surface with the underlying soft parts. Then the posterior renal chamber was uncovered, and opened with sterile scissors to expose the coiled gut (Godoy et al. 2013). Next, a small slit was opened in the first convolution of this part of the gut and the intestinal content was sampled with a sterile micropipette tip (N=3 per snail). The samples were cultured under aerobic conditions in a liquid selective medium containing uric acid as the only carbon and nitrogen source (0.02 % $MgSO_4.7\ H_20$, 0.00005 % $FeSO_4.7\ H_20$, 0.002 % $MnSO_4.7\ H_20$, 0.005 % $CaCl_2$, 0.25 % K_2HPO_4, 0.5 % KH_2PO_4, and 0.15 % uric acid; salts and uric acid were dissolved in 0.075 N NaOH and then neutralized with 10 % KH_2PO_4, according to Rouf and Lomprey, 1968). Incubations were done at 31 °C since our previous observations at different temperatures (25–37 °C) indicated optimal growth at this temperature. After repeated streaking in over 200 plates containing the selective medium (solidified with 1.5 % agar) 74 strains were obtained, but only eight were preselected as different isolates on the basis of similar colony appearance and Gram staining (Beveridge 2001).

DNA was extracted from each bacterial isolate by the phenol-chloroform method (Sambrook et al. 1989) and the 16S rRNA gene was PCR-amplified using the generalized bacterial primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525r (5'-AAGGAGGTGWTCCARCC-3'; Weisburg et al., 1991) in an Eppendorf Mastercycler Gradient thermal cycler as previously described (Olivera et al. 2009). PCR products' aliquots were used to test quality and size by 1.2 % agarose gel electrophoresis and a single band of the expected size (~1500 bp) was obtained in each case and purified (Wizard® SV Gel and PCR Clean Up System, Promega). The purified bands were sequenced (both senses) by the dideoxynucleotide method (UGB-INTA, Castelar, Argentina). The resulting sequences were analyzed with BioEdit (Hall 1999) to obtain consensus sequences for each isolate. The 16S rRNA gene sequences corresponding to bacterial isolates in this study were deposited in the GenBank under accession numbers KFO12637 to KFO12642.

Phylogenetic analysis was underwent comparing 1329 bp gene sequences corresponding to isolates from this study (Table 1) and sequences obtained from GenBank database representing related genera from Proteobacteria and Firmicutes. A phylogenetic tree was constructed using the Neighbor-Joining algorithm, from a distance matrix calculated following Kimura's two-parameter model. The stability of the clades was assessed by bootstrapping (1000 replications). Analyses were performed using the MEGA5 program (Tamura, 2011).

For the determination of uricase activity, each bacterial strain was mass cultured at 31 °C (overnight) in 200 mL of the selective medium, and then centrifuged at 10,000 g for 20 min. The bacterial precipitates were stored at −85 °C until testing for uricase activity. For the activity assay, each bacterial pellet was thawed and resuspended in 1 mL of Tris–HCl buffer (50 mM, pH 7.6), sonicated twice in an ice bath for 1 min, and centrifuged at 13,000 g for 5 min at 4 ° C. Uricase activity was determined in 20–25 μl of the supernatant fraction by incubation at 31 °C (during 30 min) with different substrate concentrations (6.25, 12.5, 25 and 30 μM uric acid) and pH values (7, 7.3, 7.6, 8.0, 8.5, 8.7 and 9.0). Enzyme specific activity was expressed in μM uric acid consumed per mg of protein and per min. For such purpose, uric acid and protein concentrations were determined as previously described (Vega et al. 2007).

Table 1 Uric acid-degrading bacterial strains isolated from the coiled gut lumen of *Pomacea canaliculata*: 16S rDNA sequences and specific uricase activity at optimal pH and substrate concentration values.

Isolate ID#	Assigned genus	GenBank accession number	pH	Substrate concentration [μmol]	Specific activity (μg/mg/min)
5C	*Pseudomonas*	KF012639	8.5	30	0.0075
8	*Pseudomonas*	KF012638	8.5	30	0.033
360	*Pseudomonas*	KF012640	7.3	25	0.01
184	Enterobacter	KF012637	8.7	30	0.045
227	*Citrobacter*	KF012641	–	–	–
230	*Lactococcus*	KF012642	7.8	30	0.028

3 Results

Among the bacterial strains selected for their ability to grow in a medium containing uric acid as the only carbon and nitrogen source, eight isolates were selected on the basis of similar colony appearance and Gram staining (Beveridge 2001). From these, 6 different 16S rRNA gene sequence were recovered, as summarized in Table 1.

A phylogenetic tree was constructed with the gene sequences obtained from the isolates on Table 1, where the GenBank accession numbers are given, and with 309 GenBank sequences representing the related genera from Proteobacteria and Firmicutes (these 309 accession numbers are given as supplementary material (S01). The phylogenetic tree indicated that isolates 5c, 8 and 360 were placed among representatives of *Pseudomonas* (Pseudomonadaceae, Fig. 1), isolates 184 and 227 were placed among *Enterobacter* and *Citrobacter*, respectively (Enterobacteriaceae, Fig. 2), and isolate 230 among *Lactococcus* (Streptococcaceae, Fig. 3).

Uricase activity was consistently determined in the *Pseudomonas*, *Enterobacter* and *Lactococcus* isolates. The optimal pH values found were 7.3-8.7, and optimal substrate concentrations were 25–30 μM (Table 1). The *Citrobacter* isolate did not show consistent activity values at different substrate concentrations.

4 Discussion

A variety of eukaryotic symbionts have been reported in snails in the family Ampullariidae (Vega et al. 2006; Hayes et al. 2014), but the associated bacteria have not been identified, except for a single report of a *Bacillus* sp. (Li et al. 2006) isolated from the gastric juice of a misidentified ampullariid

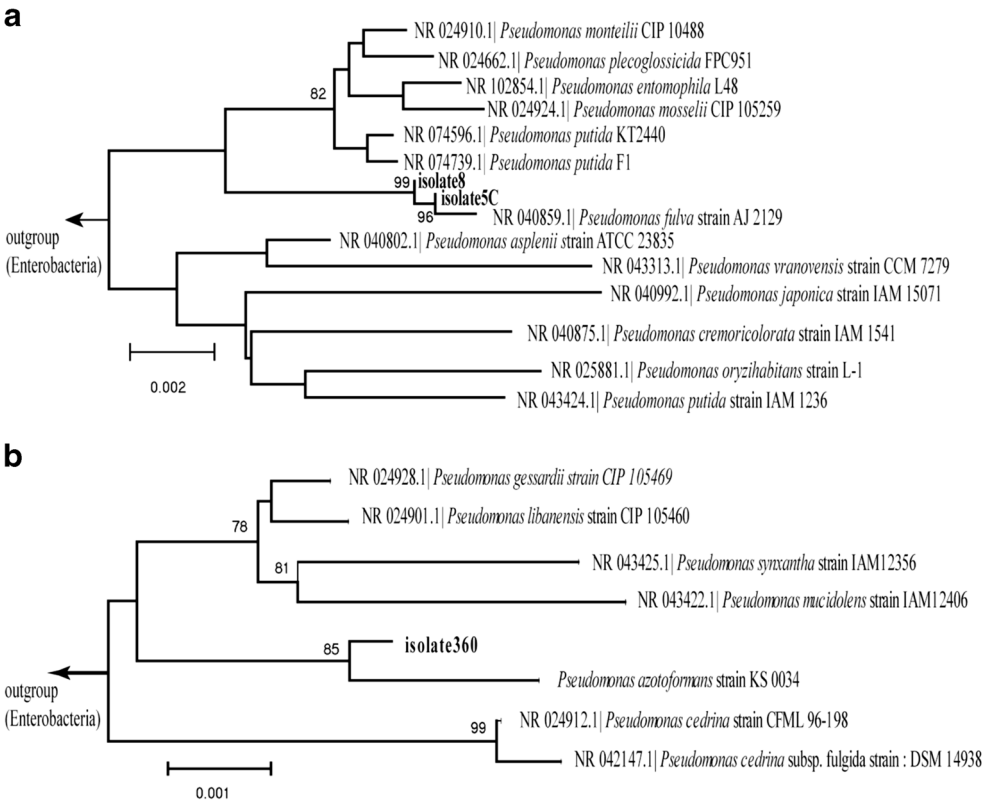

Fig. 1 Phylogenetic relationships between *Pseudomonas* 16S rRNA gene sequences with those from isolates 8 and 5C (panel *a*) and 360 (panel *b*).

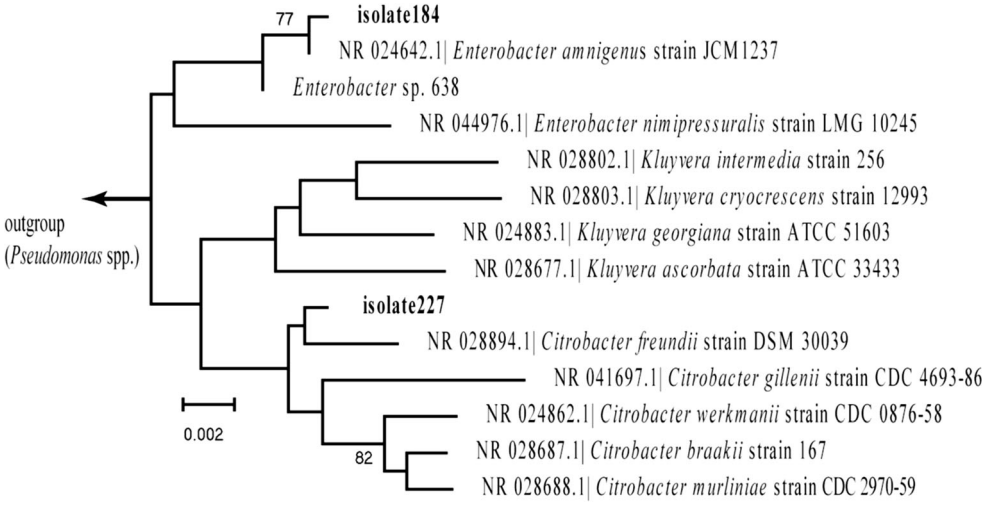

Fig. 2 Phylogenetic relationships between *Enterobacter* and *Citrobacter* 16S rRNA gene sequences with those from isolates 184 to 227.

snail, which was probably also *P. canaliculata* (Hayes et al. 2014). Also, identification of gut bacteria has been made in vetigastropods in the genus *Haliotis* (Tanaka et al. 2004; Zhao et al. 2012) and in several pulmonate snails, i.e., in taxa which are phylogenetically distant from ampullariid snails (Fig. 4). Among pulmonates, representatives of the genera *Biomphalaria* (Silva et al. 2013; Van Horn et al. 2011), *Bulinus*, *Helisoma* (Van Horn et al. 2011), *Helix* (including *Cornu*: Charrier et al. 1998; Charrier et al. 2006) and *Achatina* (Cardoso et al. 2012a, b) have been studied.

Most culture-based studies in pulmonate gastropods have used aerobic methods, with the exception of those of Charrier and co-workers (Charrier et al. 1998, 2006; Nicolai et al. 2005) in the gut of *Helix aspersa*, in which a predominant *Enterococcus casseliflavus*, and a *Clostridium* sp. have been found. However, these authors reported that bacterial counts in the gut did not differ statistically when either aerobic or anaerobic culture methods were applied to the gut of *H. aspersa* (Charrier et al. 2006). Not surprisingly, however, a much greater bacterial diversity has been disclosed by culture-independent molecular methods in the gut of *Haliotis*, *Achatina*, *Bulinus*, *Biomphalaria* and *Helisoma* species (Cardoso et al. 2012b; Van Horn et al. 2011; Tanaka et al. 2004) which have widened the scenario of possible ecological interactions among gut microbes, and which includes numerous Fungi and Viruses (Cardoso et al. 2012a). Representatives of three (*Citrobacter*, *Enterobacter* and *Pseudomonas*) of the four bacterial genera which are here reported as showing uricolytic activity (Table 1) have also been found in at least some freshwater pulmonate (Silva et al. 2013; Van Horn et al. 2011), while the fourth genus (*Lactococcus*) has also been identified in the terrestrial genera *Helix* (Charrier et al. 2006) and *Achatina* (Cardoso et al. 2012b). In no case, however, the uric acid-degrading capacity of these bacteria has been explored.

Gut bacteria in gastropods are known to participate in digestion of macromolecules, such as cellulose and hemicellulose (the main constituents of plant cell walls) and proteins. Recently, Cardoso et al. (2012a) reported numerous cellulase and hemicellulase sequences in the crop of *A. fulica*.

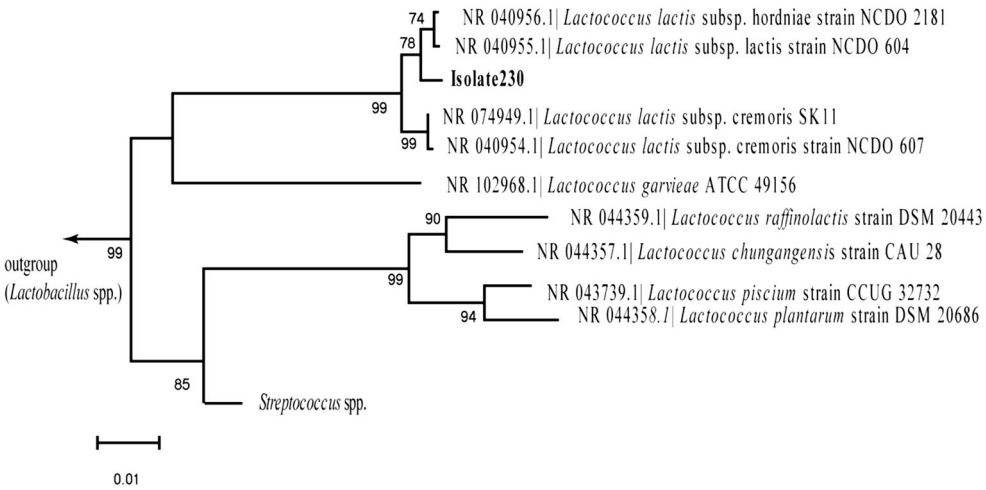

Fig. 3 Phylogenetic relationships between *Lactococcus* 16S rRNA gene sequences with that from isolate 230.

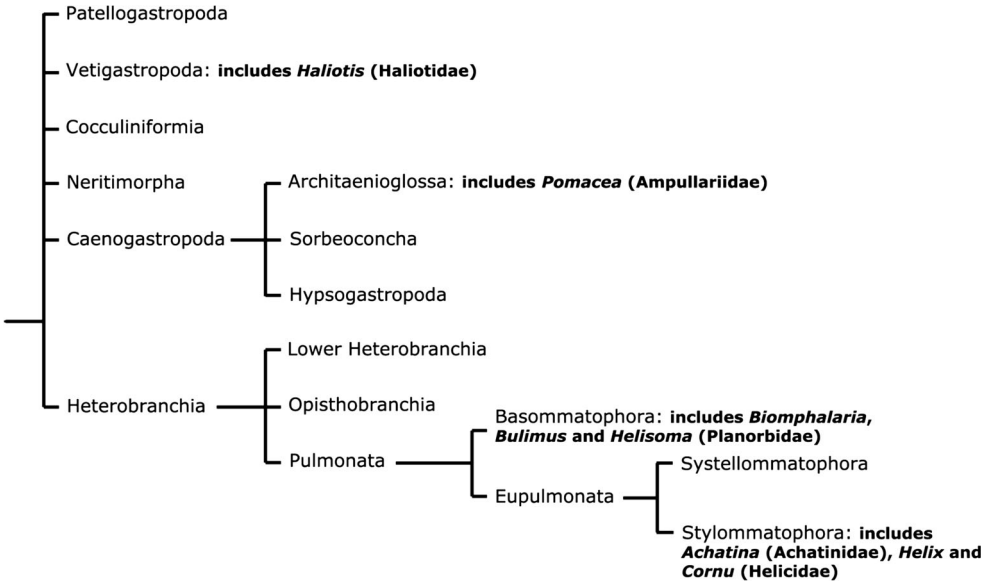

Fig. 4 Simplified high rank taxonomy of the class Gastropoda (based on Bouchet and Rocroi 2005) to illustrate relationships between the gastropod genera mentioned in the Discussion.

Specifically for *P. canaliculata*, Vega et al. (2005) observed that this snail was able to survive for at least 8 weeks on a cellulose-only diet, which indicated the existence of cellulase/s in the gut, and later, both host (Imjongjirak et al. 2008) and bacterial endoglucanases (Li et al. 2006, 2009) have been reported. However, earlier studies (Simkiss and Watkins 1990; Lesel et al. 1990) only found small numbers of cellulose-degrading bacteria in *H. aspersa* and none of the strains isolated by Charrier et al. showed this capacity (1998; 2006). Protein digestion has also been studied in gastropods (e.g., Zhao et al. 2012; Evans and Jones 1962; Cockburn and Reid 1980), but no conclusive evidence for a role of bacterial enzymes has been obtained (Walker et al. 1999) except for a 32 kDa protease present in the midgut gland and the gut lumen of *P. canaliculata*, which is produced by a prokaryotic symbiont of uncertain phylogenetic position (Godoy et al. 2013).

Besides those digestive roles, the occurrence of uricolytic gut bacteria in *P. canaliculata* may be significant for recycling of uric acid stored in specialized tissues as it occurs in insects (see Introduction). Such recycling of uric acid requires release and degradation from these tissue stores, and the first step for this should be the oxidation of this purine to allantoin, a reaction that occurs non-enzymatically (as it has been shown in *P. canaliculata*; Giraud-Billoud et al. 2011; Giraud-Billoud et al. 2013) or that it may be initiated by uricase (Lehninger et al. 2004). In the current paper, uricase activity (Table 1) could be consistently measured in extracts from 5 of the 6 isolates obtained from the gut of *P. canaliculata*. The pH values for optimal enzyme activities (Table 1) encompassed the pH values reported in the gut of *P. canaliculata* (Godoy et al. 2013) and the optimal substrate concentrations (Table 1) were similar to those reported in circulating hemolymph of this snail (Cueto et al. 2011). These findings should be considered as a first approach to a possible role of the intestinal microbiota in recycling of uric acid nitrogen in *P. canaliculata*. Since we have used an aerobic culture method, our isolates may only partially represent the uricolytic microbiota of this snail. Therefore, this approach will have to be complemented by anaerobic culture as well as by culture-independent molecular approaches, such as fingerprinting methods or 16S rRNA gene amplicon pyrosequencing. In addition, metagenomics is another approach that can reveal not only the actual diversity of gut bacterial communities, but also give insights into their metabolic potential. In addition, the actual incorporation of uric acid nitrogen into the host cells needs to be established in *P. canaliculata*.

A working hypothesis of uric acid role/s in *P. canaliculata* is presented in Fig. 5 where current knowledge and some hypothetical steps are integrated. Uric acid may come both

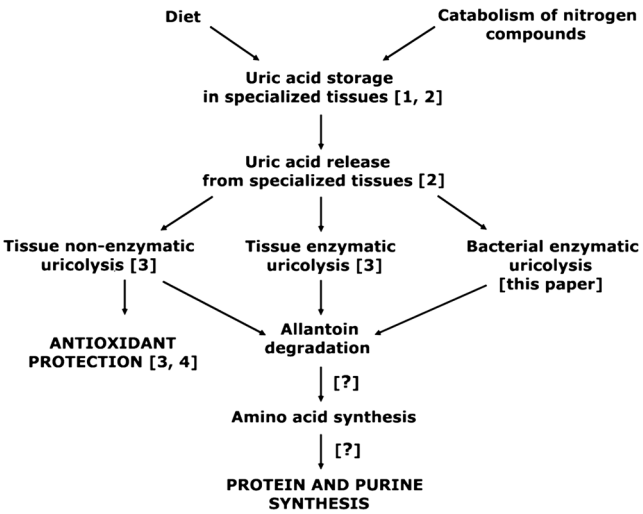

Fig. 5 A working hypothesis on uric acid roles in *P. canaliculata*. Abbreviations: [1] Vega et al. 2007; [2] Giraud-Billoud et al. 2008; [3] Giraud-Billoud et al. 2011; [4] Giraud-Billoud et al. 2013.

from the diet and from metabolism of nitrogen compounds (nucleic acids, proteins; Lehninger et al. 2004). In fact, uric acid is found in the circulation (Cueto et al. 2011) and in the renal filtrate of ampullariid snails (Little 1968), but it seems to be resorbed from the filtrate since it is not found in the excreta (Vega et al. 2007). A physiological role for uric acid in antioxidant protection seems well established, and this is done through non enzymatic degradation to allantoin (Giraud-Billoud et al. 2011, 2013). However, uricase is found in several tissues of this snail, though its activity shuts down during estivation, and it does not participate in uric acid oxidation during arousal from this state (Giraud-Billoud et al. 2011). However, tissue uricase may still play a role in nitrogen recycling in active snails, together with that of bacterial uricase.

Further studies of the possibility of nitrogen recycling by *P. canaliculata* are important since this could constitute another adaptive strategy underlying the remarkable ability of this snail to invade new habitats and to threaten several crops, notably rice (Hayes et al. 2014; Cowie 2002; Lowe et al. 2000).

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