The gut-associated *Klebsiella* sp. of the apple snail produces multiple polysaccharide degrading enzymes

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***Pila globosa*, an edible variety of apple snail is a common inhabitant of lentic ecosystem and feeds on plant detritus. The tissue extract of gastrointestinal tract from *Pila globosa* demonstrated the presence of carboxymethyl cellulase, xylanase, alginate lyase and pectinase activity. Culture-dependent method was used to isolate carboxymethyl cellulose (CMC) degrading bacteria from the gastrointestinal tract of apple snail. Morphologically identical colonies were obtained on M9 gelrite plates containing CMC as carbon source. One such bacterial isolate was purified by streaking and designated as strain PG-1. Bacterial strain PG-1 degraded CMC, pectin, starch and alginate. The 16S rDNA sequence of strain PG1 was 99% identical to *Klebsiella oxytoca*. Phylogenetic analysis using maximum likelihood revealed the clustering of strain PG1 with the clade belonging to *Klebsiella oxytoca* type strain.**

Keywords: Apple snail, *Klebsiella*, *Pila globosa*, polysaccharide degrading bacteria.

PILA GLOBOSA, commonly referred to as apple snail, inhabit lentic ecosystem and is widely distributed in Asia. Besides being a human delicacy, apple snail is used to feed shrimps and fishes[1]. The apple snail soup is also used in traditional medicine for bringing relief against asthma, tuberculosis and stomach disorders[2]. Snails feed on algal or plant detritus and reportedly degrade cellulose, laminaran, and mannan[3,4]. The capability of snails to degrade polysaccharide is linked to presence of cellulase, amylase, alginate lyase and mannanase in the digestive fluids of marine gastropods[5–10]. All molluscs persistently ingest bacteria from soil, sediments and water resulting in unique microbiota in the gut region[11]. Kiran *et al.*[12] studied bacterial diversity in different regions of gastrointestinal tract (GI) of giant african snail (*Achatina fulica*) and reported the presence of various bacterial genera including *Citrobacter*, *Kluyvera*, *Acinetobacter*, *Escherichia*, *Shigella, Salmonella*, *Acidovorax*, *Staphylococcus*, *Bacillus*, *Enterococcus*, *Lactococcus*, *Kurthia* and *Exiguobacterium* in the whole gut region. The microbiota in GI are believed to play an important role in degrading plant detritus by providing the bacterial host with a battery of polysaccharide hydrolysing enzymes (carbohydrases)[12].

In the present study, we report the isolation and identification of autochthonous multiple polysaccharide degrading *Klebisiella* sp. from the GI of *P. globosa* using culture dependent method. Furthermore, the endogenous carbohydrase activities from the extract of GI were compared with carbohydrase produced by *Klebsiella* sp.

P. globosa was collected from the paddy fields of Goa, India ($n = 10$). After washing with tap water, the snails were surface-sterilized by dipping in 70% ethanol for 8 minutes. The snails were air-dried in laminar flow hood and rinsed thrice with sterile distilled water. After removal of outer shell, GI was dissected aseptically, cut into smaller pieces and homogenized in 20 mM *Tris* Cl (pH 7.0) at 4°C using Potter Elvehjem. The homogenate was centrifuged at 17,000 *g*, 4°C for 15 min. The supernatant was saturated with 80% ammonium sulphate and the protein precipitate was collected by centrifugation. The protein pellet was resuspended in 20 mM *Tris* Cl (pH 7) and later dialysed against the same buffer for 24 h. The partially purified enzyme extract was stored at –20°C and immediately used to determine CMCase, amylase, alginate lyase, pectinase and xylanase activities by measuring the amount of reducing sugar by DNSA method[13]. Alternatively, the pieces of gastrointestinal tract were aseptically added to the sterile M9 medium containing 0.2% of CMC and grown on the orbital shaker for 72 h at 30°C ($n = 3$). The broth was serially diluted and plated on M9 medium containing 0.75% gelrite and 0.2% CMC. The plates were incubated at 30°C for 24 h to check for bacterial growth. To evaluate the degradation of CMC, pectin, starch, alginate and xylan as a carbon source, strain PG-1 was inoculated in M9 medium broth supplemented with 0.2% of individual polysaccharides. After incubation for 48 h, the culture was centrifuged and CMCase, pectinase, α-amylase, alginate lyase and xylanase activities in the culture supernatant were determined by DNSA method[13]. CMC and pectin were resuspended in 0.1 M citrate buffer (pH 5) whereas other polysaccharides were resuspended in 20 mM *Tris* Cl (pH 7). The CMCase activity was determined at 45°C whereas other carbohydrase were assayed at 30°C. Glucose was used as reference sugar. One unit is defined as micromole of reducing sugar released per minute at respective temperature.

Genomic DNA from bacterial strain PG-1 was isolated by Maloy method[14]. 16S rDNA sequence was amplified using the two universal primer 27F and 1525R. The amplified PCR product was purified using QIAquick PCR purification kit (Qiagen Inc, Hilden, Germany). Sequencing reaction was carried out in a 96 well PCR plate using Big Dye terminator cycle sequencing kit using ABI-PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). Homologous sequences similar to that of strain PG-1 were retrieved from Ribosomal

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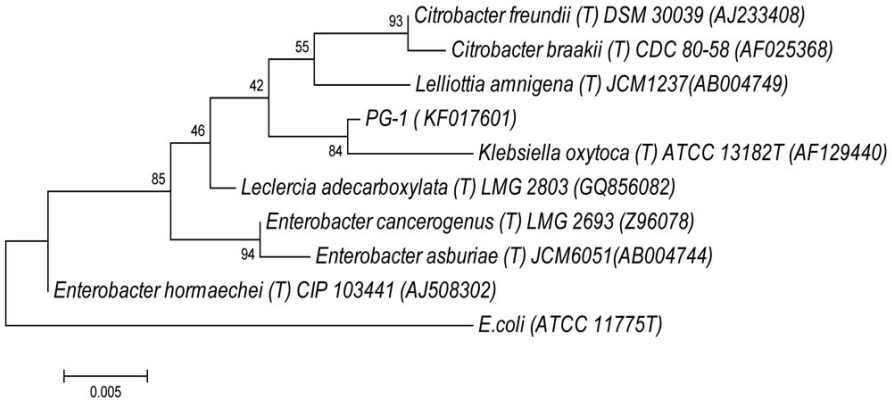

Figure 1. Maximum likelihood tree showing phylogenetic position of strain PG-1 and other closely related type strains based on 16S rDNA. Percentage of bootstrap values from 1000 replications is shown at branch points.

Table 1. Carbohydrase activities in the GI extract of *Pila globosa* and the culture supernatant of *Klebsiella* strain PG-1

Carbohydrases	Activity (U/ml)	
	GI of *Pila globosa*	Culture supernatant of *Klebsiella* strain PG-1
CMCase	0.042	0.021
Pectinase	0.057	0.0086
Alginate lyase	0.038	0.0082
Amylase	ND	0.014
Xylanase	0.034	ND

ND, Not detected.

Database Project (RDP) (http://rdp.cme.msu.edu/). Multiple sequence alignment of 16S rDNA sequence was carried out using Clustal W program. Phylogenetic tree was constructed by the maximum likelihood method using MEGA 5.1 software. Bootstrap analysis was done with 1000 replications using the bootstrap option in MEGA 5.1.

During preliminary screening for CMC degrading autochthonous bacteria from the GI tract of *P. globosa*, several bacterial isolates with identical colony morphology were predominantly and abundantly observed on M9 gelrite medium containing 0.2% CMC. One such bacterial isolate was purified by repeated streaking and designated as strain PG-1. Bacterial strain PG-1 is Gram-negative, motile and coccoid. The 16S rRNA sequence (KF 017601) of strain PG1 demonstrated sequence similarity with 16S rRNA of bacteria belonging to genus of ϒ proteobacteria in the range of 98.4–99.3%. Phylogenetic tree constructed using sequences of closely related bacterial type strains by maximum-likelihood algorithm indicated that strain PG-1 formed a coherent cluster with the clade that comprises type strains of genus *Klebsiella* and was supported by bootstrap confidence level of 84% (Figure 1). Similar tree topology was observed when trees were constructed using neighbour joining and maximum parsimony algorithms (data not shown). Thus strain PG-1 is closely related to *Klebsiella oxytoca* ATCC 13182^T and is identified as *Klebsiella* sp. strain PG-1.

Although most studies primarily report microbiota diversity in GI of snails[11,12], scanty data on polysaccharide degrading bacteria and none from GI of apple snails are available. Besides, carboxymethyl cellulase (CMCase), strain PG-1 was observed to produce carbohydrases that facilitated degradation of pectin, starch and alginate (Table 1). Although xylanase was not produced by strain PG-1, endogenous xylanase activity was independently detected in the GI extract of apple snail (Table 1) as also reported by another group[15]. Single polysaccharide degraders such as *Vibrio* strains that reportedly degrade alginate have symbiotic association with abalones and promote seaweeds digestion[16–18]. Similarly *Klebsiella* sp. observed in GI tract of giant snails degraded only CMC[19]. However, *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C from the gut of sea snail, *Batillus cornutus* were the only multiple polysaccharide degrading bacteria seemingly degrading cellulose, alginate, laminarin and kelp[20].

The GI extract of *P. globosa* produced endogenous carbohydrases that assists degradation of CMC, pectin, alginate and xylan (Table 1). Although the degradation of cellulose, pectin and starch from leaf detritus is effortlessly managed by endogenous carbohydrases produced by GI of apple snails, the carbohydrases produced by bacterial strain PG-1 possibly supplement the endogenous carbohydrase, aiding in efficient digestion of leaf detritus. Additionally, the alginate lyase production by strain PG-1 confers additional capability on apple snail to degrade plankton and algae in lentic ecosystem.

The presence of other polysaccharide degrading bacteria in GI of apple snail is being explored and the relationship of autochthonous *Klebsiella* sp. strain PG-1 with apple snail is under study.

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Application of computational methods in fish species identification based on mitochondrial DNA sequences

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The great discrepancy in sequence divergence of congeneric (0.4–0.6%) and conspecific (3%) individuals makes it difficult to identify species using DNA. A 650 base pair fragment of the cytochrome *c* oxidase subunit I (COI) gene from the fish *Pethia conchonius* was analysed using 30 samples. All the samples were identified as *P. conchonius* and two other congeneric species showing <2% sequence divergence with 29 samples out of 30 used, of *P. conchonius*. Two of the *P. conchonius* samples clustered with *Puntius terio*. Using different computational methods, we identified the sequence that was tagged as *Puntius chola* in the NCBI database as the *P. conchonius* sequence.

Keywords: Character attribute, *Puntius*, sequence divergence.

SPECIES identification is an important aspect for exploring biodiversity, wildlife forensics, ornamental trade and other fields of biology. Fishes are a highly diverse species constituting more than 50% of all vertebrates globally[1,2]. The segment near 5′-terminus of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) is frequently used in taxonomy, and has been selected as the barcode region of the entire animal kingdom[2,3]. The effectiveness of this gene has been validated in different animal groups, and

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