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ORIGINAL ARTICLE

Genome sequencing of *Pediococcus acidilactici* (NRCC1), a novel isolate from dromedary camel (*Camelus dromedarius*) rumen fluid

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Abstract

The lactic acid bacterium *Pediococcus acidilactici* has recently been reported to help in treating constipation, diarrhea, relieving stress, and enhancing growth rate and immune response in humans, birds, fishes, and small animals. In the present study, we sequenced and analyzed the whole genome of *P. acidilactici* NRCC1, a novel isolate from rumen fluid of dromedary camel (*Camelus dromedarius*). The genome of *P. acidilactici* NRCC1 was assembled into 60 contigs, comprising 1,785,679 bp and 42.5% GC content. The 1705 CDS were predicted and annotated using the RAST server. The genome encodes numerous enzymes for utilization of different carbohydrates. It also harbors genes for antibiotic biosynthesis and many others which might confer probiotic properties. The comparative genome analysis with *P. acidilactici* DSM 20284 revealed some unique features in *P. acidilactici* NRCC1. Thus, the genome sequencing of *P. acidilactici* NRCC1 has opened up new horizons for further research in animal probiotics and feed supplements.

Keywords Camel · Genome analysis · *Pediococcus acidilactici* · Probiotic · Rumen microflora

Introduction

The dromedary camel (*Camelus dromedarius*), a unique animal highly adapted to the desert ecosystem, can digest a range of plant materials, including low-quality shrubs and trees. This ability can be attributed to the extensive microbial population in the forestomach comprising bacteria, archaea, fungi, and protozoa (Bhatt et al. 2013). The digestive anatomy and physiology of the dromedary camel is different from that of true ruminants like cattle, sheep, and goat. The forestomach of camel comprises only three chambers (C1, C2, and C3), while in true ruminants, four chambers are present (Fowler 2010). Chamber C1 is a large anaerobic fermentation chamber analogous to the rumen in function and harbors a distinct microbial community that enables the camel to digest, ferment, and extract the nutrients efficiently from plant lignocellulosic material (Kay and Maloiy 1989). Studies suggest that the camel rumen microbiome is structurally similar but compositionally distinct from other ruminants (Gharechahi et al. 2015; Dande et al. 2015). At the phylum level, Firmicutes comprises the second largest group, accounting for about 31% of the total bacterial population in camel rumen (Gharechahi et al. 2015).

Pediococcus acidilactici is a homofermentative, Gram-positive, nonmotile, catalase-negative facultative anaerobe belonging to phylum, Firmicutes; class, Bacilli; order, Lactobacillales; and family, Lactobacillaceae. It can grow in a wide range of pH, temperature, and osmotic pressure and can colonize the digestive tract of humans and animals (Klaenhammer 1993). Besides, it is also present in fermented vegetables, fermented dairy products, and meat. They possess several beneficial health effects, helpful in treating constipation, diarrhea, relieving stress, and enhancing growth rate and immune response in birds, fishes, and small animals (Ferguson et al. 2010). Some strains isolated from food and

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the human gastrointestinal system have recently been tested as a probiotic supplement and found to prevent colonization of pathogens such as *Shigella* spp., *Salmonella* spp., *Clostridium difficile*, and *Escherichia coli* in the small intestine (Feng et al. 2016). *Pediococcus acidilactici* produces distinct pediocins that are active against a broad spectrum of Gram-positive bacteria (Cintas et al. 1995). To our knowledge, this is the first study documenting the isolation of *P. acidilactici* from camel rumen fluid and its genetic analysis. The draft genome sequence of *P. acidilactici* NRCC1 was analyzed with particular reference to its probiotic potential and functional characteristics were compared with *P. acidilactici* DSM 20284.

Materials and methods

Rumen fluid collection

A rumen fluid sample was collected from an 8-year-old healthy male dromedary camel (*Camelus dromedarius*) using a rumen fluid extraction unit designed for camels as described elsewhere (Bhatt et al. 2013). The first 200-mL sample obtained was discarded to avoid contamination with saliva. Thereafter, rumen contents (50 mL) was collected and filtered through four layers of autoclaved gauze, and the filtered rumen fluid was used for microbial culture and isolation. The animal was maintained under an intensive system of management and fed daily with guar (*Cyamopsis tetragonoloba*) meal (mixture of 30–33% hull, 27–30% endosperm, and 43–47% germ) and groundnut (*Arachis hypogaea*) haulms. Ethical standards and guidelines as recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India and as approved by the Institute Animal Ethics Committee were followed throughout during animal handling for sample collection.

Microbial culture and isolation

The rumen fluid was processed for culturing as described by Mah and Smith (2009). Briefly, the samples was cultured on the modified Mah et al. (1978) medium containing starch 1 g, yeast extract 0.25 g, K_2HPo_4 0.04 g, $MgCl_2$ 0.01 g, $MgCl_2$ 0.01 g, $NaHCo_3$ 0.05 g, cysteine hydrochloride 0.01 g, peptone 0.25 g, mineral solution 5 mL, vitamin solution 0.5 mL, and distilled water 100 mL. The culture was incubated anaerobically at 38 °C for 48 to 72 h. A mixture of nitrogen and carbon dioxide (80:20) was purged continuously during the incubation to maintain anaerobic conditions. Repeated subculturing was done on the same media until pure culture was obtained. The isolated pure culture was submitted to the rumen microbes repository of the National Centre for Veterinary Type Culture Collection, ICAR-National Research Centre on Equines, Hisar, Haryana, India with accession no. VTCCRM0000259B.

Biochemical analysis for utilization of different sugars

The API 20A test kit (bioMérieux, La-Balme-les-Grottes, France) was used to test the carbon utilization profile of *P. acidilactici* NRCC1, as per the manufacturer's instructions. Strict anaerobic conditions were maintained using gas phase N_2:CO_2 (80:20) and the glovebox was maintained in anaerobic conditions with N_2:H_2:CO_2 (80:10:10) during the incubation. The results of the biochemical tests were later compared with genome features for the presence of genes coding specifically for sugar metabolism.

Genomic DNA isolation and sequencing

A pure culture with uniform colony characteristics and morphology was processed for DNA isolation. The genomic DNA was isolated using a commercial DNA isolation kit (GenElute Bacterial Genomic DNA Kit, NA2110; Sigma-Aldrich) and the concentration was measured using a NanoDrop Spectrophotometer ND1000 (Thermo Scientific, USA). The whole genome was sequenced using Ion Torrent PGM. The library was prepared using the Ion Plus Fragment Library Kit by following the manufacturer's instructions. In brief, the genomic DNA was fragmented and adaptors were ligated. The desired size of library fragments was selected using the E-Gel SizeSelect kit (Thermo Fisher Scientific, USA), subjected to emulsion polymerase chain reaction (PCR), and sequencing by Ion PGM using the 318 Chip.

Genome assembly and annotation

PRINSEQ (Schmieder and Edwards 2011) was used for quality filtering, where sequences with mean quality score < 20 and sequences shorter than 40 bp were filtered out. The remaining good-quality reads were mapped to *P. acidilactici* DSM 20284 using GS Reference Mapper (Newbler) v2.3. The assembled genome was uploaded to the RAST server for feature prediction and annotation. The whole genome shotgun sequence has been deposited in DDBJ/EMBL/GenBank under the accession number LQNQ00000000 and the version described in this paper is version LQNQ01000000.

The reference genome of *P. acidilactici* strain DSM 20284 was processed with the same pipeline (RAST). CGView Server (Grant and Stothard 2008) was used to show the physical map of the genome. Further, we also calculated the average nucleotide identity (ANI) between these two genomes using the ANI calculator (Goris et al. 2007) and the Orthologous Average Nucleotide Identity Tool (OAT) (Lee et al. 2016). Benchmarking Universal Single-Copy Orthologs (BUSCO) v2 (Simão et al. 2015) was used to check the

completeness of the assembled genome. tRNAscan-SE (Lowe and Eddy 1997) was used to identify tRNAs in the genome.

Metabolic features

The carbohydate-active enzyme profiles of *P. acidilactici* NRCC1 and reference strain DSM 20284 were compared using the Carbohydrate-Active Enzymes (CAZy) database (Lombard et al. 2014). A Pfam-based sequence annotation of the predicted amino acids gene sequences of both the genomes was performed using the CAZymes Analysis Toolkit (Park et al. 2010) with minimum E value 1E-5. Enzymes and pathways information was retrieved using KEGG analysis. antiSMASH (Medema et al. 2011) was used to identify secondary metabolite biosynthesis gene clusters.

Phylogenetic analysis

A BLASTn search of the assembled genome was carried out against 16S ribosomal RNA sequences of bacteria and archaea to identify the organism. The 16S rRNA gene sequence of *P. acidilactici* was submitted to the NCBI with accession no. KU504251. For phylogenetic analysis, all of the 16S rRNA gene sequences of *P. acidilactici* with size > 1.5Kb were downloaded from the NCBI. Sequences were aligned using MAAFT (Katoh et al. 2012) and poorly aligned regions were removed using the Gblocks server (Talavera and Castresana 2007). Thereafter, the neighbor joining (NJ) tree was constructed using MEGA (Molecular Evolutionary Genetics Analysis) version 7.0 (Kumar et al. 2016). The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site.

Results

Colonies of *P. acidilactici* NRCC1 appeared as small, round, milky-white, and opaque, with irregular margins and smooth moist surface. The organism after Gram staining appeared as Gram-positive, long, single rods.

Biochemical properties and relative enzyme encoding CDS in the genome

After removing low-quality sequences (Phred score < 20 and minimum length 40 bp), a total of 1,951,776 sequences (493,599,611 bp) were subjected to mapping against the *P. acidilactici* DSM 20284 (RefSeq ID NZ_CP015206.1) genome using Newbler v2.6. From these, a total of 1,374,981 sequences corresponding to 353,510,036 bases were mapped against the reference genome with 60 consensus contigs. The genome comprises of 1,785,679 bases with 42.5% GC content. The genome of *P. acidilactici* NRCC1 was sequenced with 198X coverage. The genome features of *P. acidilactici* NRCC1 are presented in Table 1 and Fig. 1 shows the physical map of the genome.

At the phyla level (Firmicutes), BUSCO revealed 226 complete and single-copy BUSCOs, which correspond to 97.5% completeness, while at the order level, a total of 428 single-copy BUSCOs were present, corresponding to 96.6% completeness of the genome (Supplementary Table 1). tRNAscan-SE identified a total of 52 tRNAs in the *P. acidilactici* genome, encoding for 20 different amino acids (Supplementary Table 2).

Functional annotation

RAST annotation predicated a total of 1705 CDS (Supplementary Table 3) and, of these, 1013 were functionally classified with subsystems (Fig. 2a and Supplementary Table 4). A large proportion of the CDS were classified into the carbohydrate and protein metabolism. A total of 172 CDS of *P. acidilactici* NRCC1 was found to participate in the carbohydrate metabolism. The feature was further classified into enzyme coding for subcategories, such as the central carbohydrate metabolic pathways (43), monosaccharides (54), di- and oligosaccharides (37), fermentation (13), amino sugars (8), organic acids (6), sugar alcohols (6), one-carbon metabolism (4), and carbohydrates, no subcategory (1). The total number of CDS participating in the carbohydrate metabolism in *P. acidilactici* DSM 20284 was 183, with an almost similar pattern of enzyme coding for subcategories like central carbohydrate metabolic pathways (43), monosaccharides (54), di- and oligosaccharides (38), fermentation (13), amino sugars (10), organic acids (14), sugar alcohols (6), one-carbon metabolism (4), and carbohydrates, no subcategory (1). Likewise, the protein metabolism profiles of the

Table 1 General features of the *Pediococcus acidilactici* NRCC1 genome

Feature	Value
Total bases	1,785,679
Total contigs	60
Maximum contig length (bp)	188,345
Mean contig length (bp)	29,761
Minimum contig length (bp)	295
GC content (%)	42.5
Coverage	198X
N50	78,213
N75	50,987
N90	17,876
N95	7995
CDS	1705
tRNAs	52
CAZy domains	335

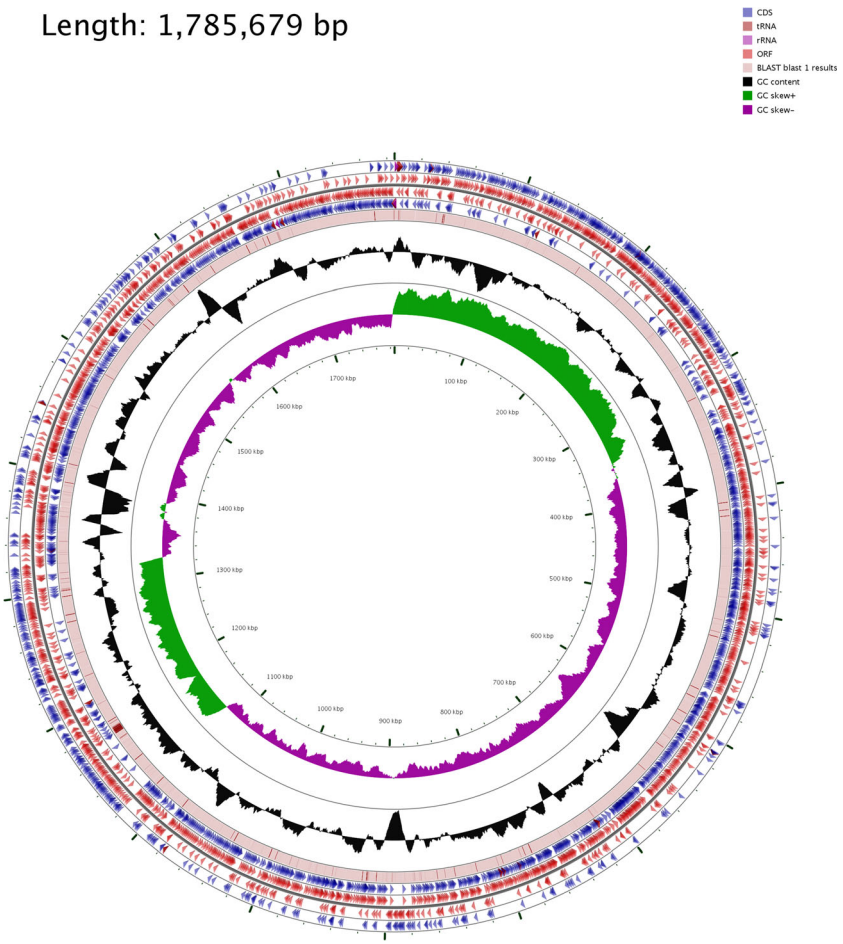

Fig. 1 Circular genome map of *Pediococcus acidilactici* NRCC1

two strains were similar, with the only difference being in the lower number of CDS present in NRCC1 for protein biosynthesis (120 vs. 150). There were 23 unique features in *P. acidilactici* NRCC1 in comparison to *P. acidilactici* DSM 20284 when all annotations of RAST were considered (Fig. 2b). Moreover, the ANI was 99.13% and 99.18% using the ANI calculator (Supplementary Fig. 1) and OAT v0.93, respectively.

Metabolic features

From the predicted amino acid sequences in the *P. acidilactici* genome, 335 putative sequences harbor domains for the CAZy family of enzymes (Supplementary Table 5). Comparative analysis of the carbohydrate-active enzyme profile of *P. acidilactici* NRCC1 revealed the presence of an equal number of enzymes in

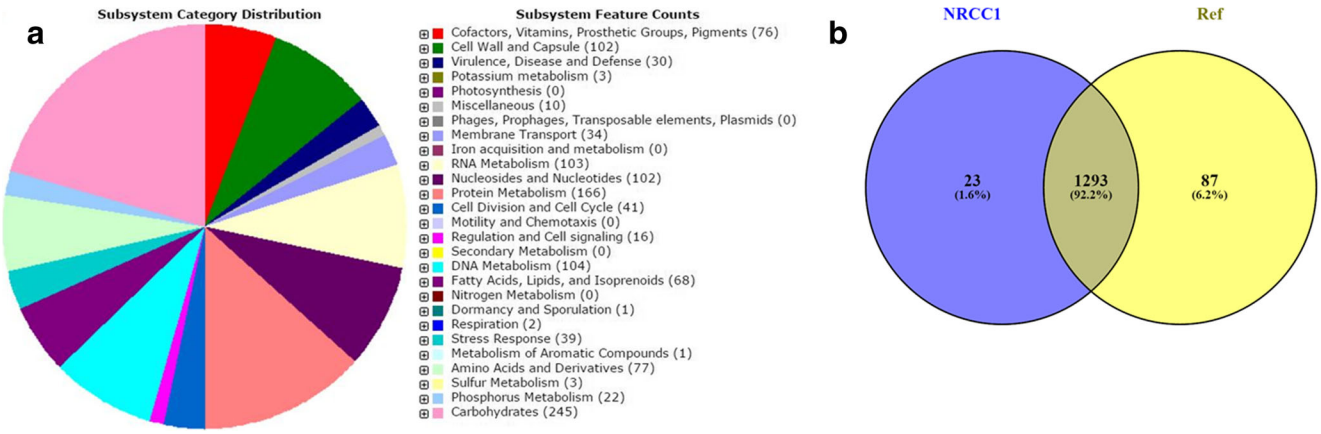

Fig. 2 **a** The COG functional classification of the *P. acidilactici* NRCC1 genome. The number of genes associated with different functional categories is shown in parentheses. **b** The comparison of *P. acidilactici* NRCC1 genomic features with reference strain *P. acidilactici* DSM 20284

the carbohydrate esterase (CE) class, but a slightly lower number of enzymes in the glycoside hydrolases (GH) and glycosyltransferases (GT) classes found in the DSM 20284 strain (Fig. 3). A total of 712 different Pfam domains are present in the genome. Further, we identified a total of 11 secondary metabolite biosynthesis gene clusters in the genome (Supplementary Fig. 2) using the antiSMASH database. One of them is the fusaricidin biosynthesis gene cluster (25% of genes showed identity), which is a peptide antibiotic (Supplementary Fig. 3). KEGG analysis showed that 1045 (61.3%) of 1705 CDS were annotated, where genetic information processing was at the top, followed by environmental information processing, carbohydrate metabolism, unclassified, and others (Supplementary Fig. 4 and Supplementary Table 6). With KEGG, 29 hits for peptidases (ko01002) were identified where serine, metallo, and cysteine peptidases were higher in count (Supplementary Table 7). A total of 73 KOs were assigned to the biosynthesis of antibiotics. The pathway for anaerobic metabolism of carbohydrate was highlighted, where the pathway showed the formation of pyruvate mostly via glycolysis (Supplementary Fig. 5) and subsequent anaerobic fermentation. Enzymes related to pyruvate (Supplementary Fig. 6), butanoate (Supplementary Fig. 7a), and propanoate metabolism (Supplementary Fig. 7b) were also identified. The overall carbon metabolism pathway in *P. acidilactici* NRCC1 is shown in Supplementary Fig. 8.

Biochemical analysis for utilization of different sugars

Biochemical tests for the fermentation profile using API 20A kits and roll tubes showed positive results for the fermentation of nine sugars, including arabinose, galactose, glucose, glycerol, esculin, mannose, rhamnose, trehalose, and xylose. Genome analysis confirmed the presence of genes coding for enzymes involved in the metabolic pathway for utilizing these sugars (Table 2). Gene sequences for metabolism of sugars like mannitol, melezitose, esculin, gelatin, and raffinose were absent, which corroborated the negative results as obtained in the API 20A test kits and roll tubes.

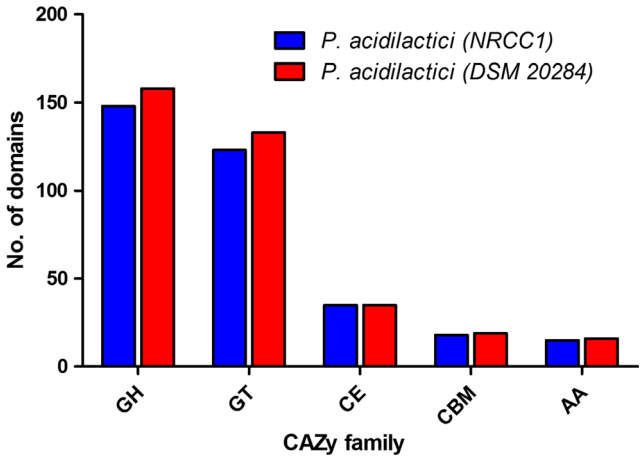

Fig. 3 Comparison of the carbohydrate-active enzyme profiles of *P. acidilactici* NRCC1 and *P. acidilactici* DSM 20284. GH stands for glycoside hydrolases (hydrolysis and/or rearrangement of glycosidic bonds), GT for glycosyltransferases (formation of glycosidic bonds), PL for polysaccharide lyase, CE for carbohydrate esterase (nonhydrolytic cleavage of glycosidic bonds), CBM for carbohydrate-binding module (adhesion to carbohydrates), and AA for auxiliary activities (redox enzymes that act in conjunction with CAZymes)

Phylogenetic analysis

BLASTn search against bacterial and archaeal 16S rRNA databases revealed that contig number 58, which is of 1783 bp, showed 99% similarity with the complete 16S rRNA gene sequence of *P. acidilactici* strain DSM 20284 (1569 bp), with query position 66–1634 bp. As shown in Fig. 4, the isolate *P. acidilactici* NRCC1 is more closely related to the *P. acidilactici* strain SM1 (KX688797.1), which was isolated from fermented moong in Gujarat, India. The second close neighbor is *P. acidilactici* strain JFP1 (KM062019.1), which was isolated from traditional food in Jeju, South Korea.

Discussion

The rumen (C1 chamber) of the dromedary camel is inhibited by a high density of resident microorganisms, including bacteria, protozoa, archaea, and fungi, which play a vital role in degradation and digestion of the ingested plant materials. In fact, the camel is more efficient in the digestion of the fiber of range plants, fodder, and grasses than other domestic ruminants (Holzapfel et al. 2009).

Pediococcus acidilactici is an important lactic acid bacteria used as starter cultures in meat, vegetable, and dairy fermentation, causing characteristic flavor changes, improving hygienic quality, and extending the shelf life of products. A range of bacteriocins (pediocins) have been identified from *P. acidilactici* strains, which, in conjunction with organic acids (such as lactic and acetic acids), result in antagonistic properties against a range of Gram-positive and Gram-negative bacteria (Ferguson et al. 2010). Some strains of the organism, like *P. acidilactici* MA18/5M isolated in France from natural-pasture Gramineae, are commercially available probiotics widely used in swine, poultry, aquaculture feeds, and human dietary supplements. In the present study, we sequenced and assembled the genome of *P. acidilactici* NRCC1 which was isolated from camel rumen fluid. The organism was able to utilize various sugars as the carbon source. This is likely because several fibrolytic rumen bacteria hydrolyze complex plant fibers and release the monomers which are subsequently utilized by other rumen bacteria. Not only the biochemical tests data but genome analysis also showed the presence of CDS encoding enzymes needed for the utilization of various sugars. Moreover, the presence of tRNAs for all the amino

Table 2 Results of sugar fermentation tests vis-à-vis coding sequences for enzymes involved in utilizing various sugars as the carbon source, present in *P. acidilactici* NRCC1

S. no.	Sugar	API 20A result	Roll tubes	CDS for enzyme(s) involved in carbon source utilization
1.	Arabinose	+	Not included	L-ribulose-5-phosphate 4-epimerase, ribulokinase, L-arabinose isomerase
2.	Galactose	Not included	+	6-Phosphofructokinase, galactosamine-6-phosphate isomerase
3.	Glucose	+	Not included	Glucose-6-phosphate 1-dehydrogenase, glucose 1 dehydrogenase
4.	Glycerol	+	Not included	2-Hydroxy-3-oxopropionate reductase, pyruvate kinase, glycerate kinase
5.	Esculin		+	Beta-glucosidase
6.	Mannose	+	Not included	Alpha-mannosidase, phosphomannomutase, mannose-6-phosphate isomerase
7.	Rhamnose	+	Not included	Rhamnulose-1-phosphate aldolase, rhamnulokinase, L-rhamnose isomerase, alpha-L-rhamnosidase
8.	Trehalose	+	Not included	Trehalose-6-phosphate hydrolase, trehalose-specific IIB component
9.	Xylose	+	Not included	Xylulose-5-phosphate phosphoketolase

acids suggest that *P. acidilactici* can synthesize all the amino acids on its own.

BUSCO is widely used to access the assembly and completeness of the assembled genome (Simão et al. 2015). Based on the presence of universal single-copy orthologous genes, the genome of *P. acidilactici* was found to be ~ 97% complete. This study revealed that the genome size and GC content of *P. acidilactici* NRCC1 and *P. acidilactici* DSM 20284 are

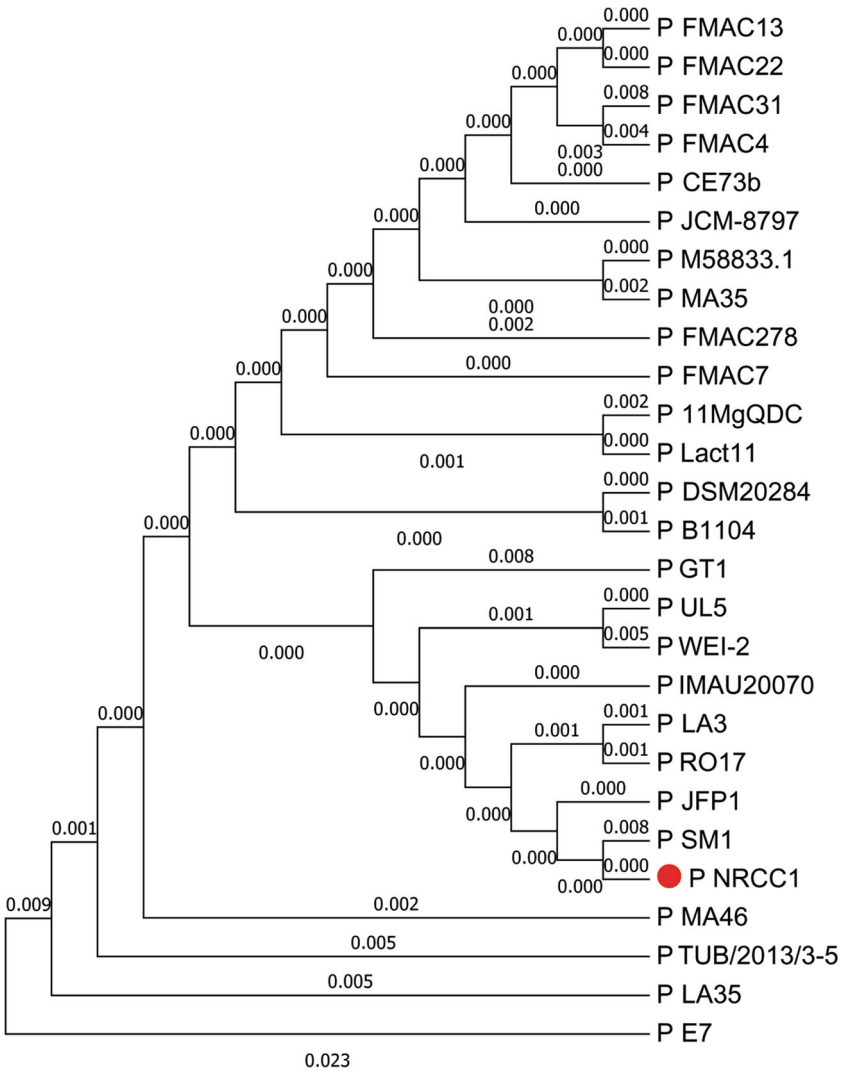

Fig. 4 Phylogenetic tree based on 16S rRNA sequences of *P. acidilactici*

similar and within the normal range reported in the literature for most of the *P. acidilactici* strains (Holzapfel et al. 2009; Barreau et al. 2012). The carbohydrate-active enzyme profiles of these two strains were also similar, though a lower number of enzymes in glycoside hydrolases and glycosyltransferases were recorded. This may be perhaps due to the larger amount of genomic data available for the reference strain. The CAZyme analysis profile further supports the results revealed by the RAST CDS prediction that the organism possesses the potential to digest various plant polysaccharides.

Different sequences coding for lactose uptake, adherence, resistance to oxidative stress, and folate synthesis were present, suggesting the probiotic potential of *P. acidilactici* NRCC1 (Prajapati et al. 2013). Resistance to acid and oxidative stress are important criteria for sustained survival of the organism in the gastrointestinal tract. Moreover, the presence of antibiotic genes as revealed by KEGG analysis as well as the presence of the fusaricidin biosynthesis gene cluster also suggests a probiotic property. Fusaricidin is a peptide antibiotic isolated from several soil bacteria effective against both fungi and Gram-positive bacteria (Choi et al. 2008; Yu et al. 2012). The fusaricidin-like gene cluster might be responsible for the biosynthesis of pediocins, which are bacteriocins produced by several lactic acid bacteria (Papagianni 2003). Pediocins from pediococci have been isolated and characterized (Porto et al. 2017). It has been observed that probiotic bacteria can modulate the rumen microbiota and improve health and productivity (Qadis et al. 2014; Uyeno et al. 2015; Abd El-Tawab et al. 2016).

Phylogenetically, *Pediococcus* and *Lactobacillus* form a supercluster that can be divided into two subclusters; all species of *Pediococcus* fall within the *Lactobacillus casei–Pediococcus* subcluster. Pediococci are morphologically distinct from lactobacilli (rods). Pediococci are only acidophilic, homofermentative, lactic acid bacteria that divide alternatively in two perpendicular directions to form tetrads (Simpson and Taguchi 1995). Phylogenetic analysis of *P. acidilactici* NRCC1 based on the full-length 16S rRNA gene sequence revealed that it is closely related to the *P. acidilactici* strain SM1 (KX688797.1) and *P. acidilactici* strain JFP1 (KM062019.1) that were isolated from fermented moong in India and traditional food in South Korea, respectively. It seems that close genetic resemblance between *P. acidilactici* NRCC1 and *P. acidilactici* strain SM1 may be due to the proximity of their isolation sites. Further, comparative analysis of the NRCC1 genome with *P. acidilactici* DSM 20284 revealed that the ANI among both the genomes was > 99%. However, while comparing RAST annotations, there were 23 unique features in the NRCC1 genome which were not annotated in DSM 20284. Similarly, 87 features were annotated in DSM 20284 but missing in NRCC1. This shows that, though the nucleotide similarity between the two strains is larger, the metabolic features of the two strains might be somewhat dissimilar, as they both arise from two different niches.

Conclusion

This is the first study reporting the isolation and genomic sequencing of *Pediococcus acidilactici* NRCC1 from dromedary camel rumen. The genomic analysis of the organism revealed that it can utilize different carbohydrates, biosynthesize antibiotics, and have several unique features suggesting its possible use in probiotics and feed supplements in animals.

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Compliance with ethical standards

Conflict of interest None.

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