

## A Reported Antarctic Environmental Microorganism Isolated from Mongolian Plateau

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### Abstract

Herein, we report a novel *Carnobacterium*-like organism, CS13<sup>T</sup>, isolated from Mongolian Plateau is 100% related to the reported Antarctic environmental microorganism *Carnobacterium antarticum* sp. CP1. The CS13<sup>T</sup> was isolated from 78 blood samples of 324 sheep with persistent diarrhea from a grassland pasturing area in Xilingol League, Inner Mongolia Municipality, China. Homology analysis indicated that CS13<sup>T</sup> belongs to the genus *Carnobacterium* and the closest relatives are *Carnobacterium antarticum* sp. CP1 (100%), *Carnobacterium mobile* DSM 4848 (97%) and *Carnobacterium funditum* DSM 5970 (96%). Similar to those of the *C. CP1*, the short rod-shaped cells of CS13<sup>T</sup> are 0.4-0.8 µm wide and 1.0-1.5 µm long; exist singly, paired or catenoid; are gram positive, non-spore forming, and facultatively anaerobic; and produce hemolysin. CS13<sup>T</sup> cannot produce gas or H<sub>2</sub>S but can ferment sucrose, galactose, salicin, and esculin to produce acid. However, in contrast to *C. CP1*, CS13<sup>T</sup> can produce acid from cellobiose and maltose and is weakly positive for D-mannose fermentation; the growth temperatures range from 20-37°C, the pH range is 5.0-9.0, and the G+C content is 37.84% (4-36°C, pH 6.0-9.5, and 38.1% for *C. CP1*). Furthermore, based on gene annotation analysis, we found that CS13<sup>T</sup> has 31 more specific genes than *C. CP1* (133 to 102) and that the nonredundant protein similarity to *C. CP1* is only 84.2%. Based on the physiological-biochemical and genetic analysis results, the organisms isolated from the Mongolian Plateau and sandy soil in Antarctica belong to the same novel species of the genus *Carnobacterium*. However, the relationship between CS13<sup>T</sup> and animal disease needs further study.

**Keywords:** Environmental Microorganism, *Carnobacterium*, Diarrhea, Sheep, Mongolian Plateau.

### Introduction

Carnobacteria are ubiquitous lactic acid bacteria (LAB), tolerant to freezing/thawing and high pressure and able to grow at low temperatures [1]. The genus belongs to the family *Carnobacteriaceae* of the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, as described in *Bergey's Manual of Systematic Bacteriology* [2], and includes motile, psychrotolerant, short rod-shaped, gram-positive, facultatively anaerobic, heterofermentative lactic acid bacteria that can produce L-lactic acid from mostly fermented D-glucose [3]. At the time of writing,

### Article Information

**Article Type:** Research

**Article Number:** JAMBR141

**Received Date:** 19 October, 2020

**Accepted Date:** 04 December, 2020

**Published Date:** 04 January, 2021

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**Citation:** Cao Z, Chen Q, Aizezi M, Li C, Zhu M, et al. (2021) A Reported Antarctic Environmental Microorganism Isolated from Mongolian Plateau. J Appl Microb Res. Vol: 4 Issu: 1 (01-06).

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12 recognized species had been correctly named and collected in the List of Prokaryotic names with Standing in Nomenclature (LPSN) collection (<http://www.bacterio.net>).

The species *C. divergens*, *C. gallinarum* and *C. mobile* are frequently encountered in the environment and in foods. *C. antarcticum*, *C. alterfunditum*, *C. funditum* and *C. iners* were isolated from sandy soil, anoxic lake water and the littoral zone of Antarctica [2,4,5]. *C. inhibens* and *C. maltaromaticum* were found in Atlantic salmon and infected Lake Whitefish, respectively [6,7]. Additionally, *C. pleistocenium* was isolated from permafrost of the Fox Tunnel in Alaska [8], *C. viridians* was isolated from vacuum-packed bologna sausage [9], and *C. jeotgali* was isolated from a Korean traditional fermented food [10]. Although a large number of research studies have reported isolation of these bacteria from various regions and environments, many species have not yet been allocated to known species, such as the *Carnobacterium*-like organisms isolated from the larval midgut of a moth species [11], spent mushroom compost [12] and watershed polluted with horse manure [13].

In this study, we isolated a novel *Carnobacterium*-like organism, designated CS13<sup>T</sup>, from the blood of sheep with persistent diarrhea in the Mongolian Plateau in China. To further clarify the diversity of this novel isolated strain and *Carnobacterium antarcticum* sp. CP1, this paper discussed the similarities and differences through culture characteristics, phenotypic characterization, and physiological-biochemical and phylogenetic characteristics.

## Materials and Methods

### Ethics statement

This study was approved by the Animal Ethics Committee of Chongqing Academy of Animal Sciences. The protocol of blood sample collection was established according to A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes [14].

### Collection of blood samples and isolation of strains

Grazing sheep with persistent diarrhea were found at Zhenglan Banner, Xilinguole League, Inner Mongolia Municipality, China (N42°42'09", E116°13'19"), in 2019. Cervical vein blood samples of sheep were collected by a sterile syringe, and the samples were stored in anticoagulative tubes at 4°C. To culture and separate the pathogens from blood samples, brain heart infusion broth liquid (BHI) medium and Columbia blood agar (CBA, supplemented with 5% (v/v) defibrinated sheep blood) medium were prepared as previously described [15]. Aliquots of 100 µL of the blood samples were streak-inoculated on CBA medium at 4°C, 20°C, 25°C, 30°C or 37°C in the presence or absence of oxygen. Colonies were observed after 72 h, and the clearest colonies were subcultured into BHI medium and then cultured for 48 h. Recovered pure cultures were preserved at -80°C in BHI broth supplemented with 20% glycerol.

### Physiology and biochemistry observation

To define the optimal culture conditions, the isolated strain was inoculated in BHI medium with extra NaCl

concentrations of 1.0-10.0% (at intervals of 1.0%, w/v) at pH 5.0-10.0 (at intervals of 1.0) at growth temperature for 72 h separately. CBA was used as a growth and hemolysin examination medium to culture the isolated strain. Gram staining was conducted with a gram staining kit (Solarbio) and observed by optical microscopy (Nikon). The morphology, size and flagellum ultrastructure of the isolated strain were observed by a JEOL JEM-1200EX electron microscope after uranyl acetate and citromalic acid lead double staining. The biochemical properties, including glycolysis reaction, indole production, hydrogen sulfide production, methyl red test, pyruvate utilization, nitrate reduction and acid production, were determined using a Micro-Biochemical Identification Tube (Hopebio).

### Homology and phylogenetic analyses

The genomic DNA of the isolated strain was extracted using a Bacterial DNA Kit (TIANGEN) and then submitted to Sangon Biotech (Shanghai) for sequencing. Homologous sequences were compared with NT (NCBI nucleotide sequences database), NR (NCBI nonredundant protein sequences database) and Swiss-Prot (manually annotated and reviewed protein sequences database). Phylogenetic analysis was performed via maximum-likelihood, maximum-parsimony and neighbor-joining algorithms in MEGA version 7.0 [16]. Additionally, comparisons of the core genes, dispensable genes and specific genes were also used in phylogenetic analyses.

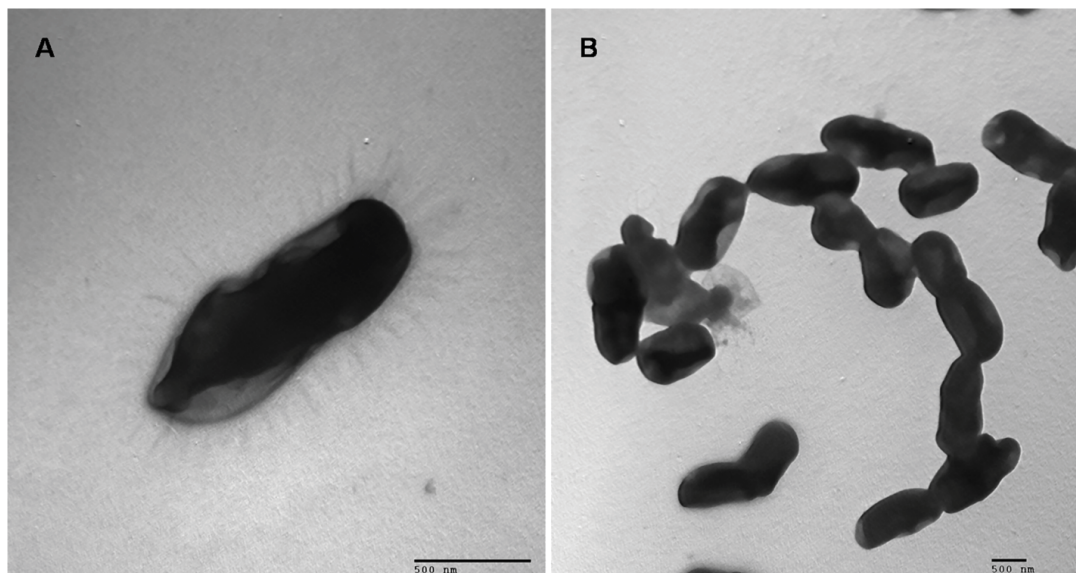
## Results

### Isolation and identification

Earlier isolates on CBA medium incubated with oxygen at 20-37°C (optimum, 30°C) for 72 h presented bacterial colonies 1-2 mm in diameter that were white-gray and opaque, with neat edges; had a smooth convex elevation and were surrounded by a tiny hemolysis halo. Furthermore, the growth of bacteria was observed at pH 5.0-9.0 (optimum pH=8.0) and in the presence of 0-5% (w/v) NaCl (optimum, 1%) when the isolated strain was inoculated in BHI media with different pH and salinity values. Electron microscopy demonstrated that the cells of the isolated strain were slightly curved short rods approximately 0.4-0.8 µm wide and 1.0-1.5 µm long, with flagella occurring singly or in pairs or short chains (Figure 1).

### Physiology and biochemistry

The isolate CS13<sup>T</sup> has biochemical and physiological characteristics similar to those of the Antarctica-isolated strains *Carnobacterium antarcticum* sp. CP1 and *Carnobacterium funditum* DSM 5970 and the frozen meat-isolated strain *Carnobacterium mobile* DSM 4848. The four strains exhibit short rod shapes, positive Gram staining, motility, facultatively anaerobic growth, growth at low temperatures, negative oxidase and catalase activities, and no H<sub>2</sub>S production. In contrast, *C. DSM 4848* is the only strain that produces gas, and except *C. DSM 5790*, all of them utilized esculin, D-glucose, D-mannose, N-acetyl-glucosamine, salicin and sucrose to produce acid. The physiological similarity between the isolates and their closest relatives among the genus are presented in Table 1.



**Figure 1: Electron Microscope Observation.** Negative stain electron microscopy of single (A) and pairs (B) of isolated strains.

## Homology analysis

The 16S rRNA gene of CS13<sup>T</sup> was sequenced by Sangon Biotech (Shanghai). 16S rRNA sequence alignment (NCBI blastn) showed that CS13<sup>T</sup> shares 100% identity with *Carnobacterium antarticum* sp. CP1, 97% with *Carnobacterium mobile* DSM 4848 and 96% with *Carnobacterium funditum* DSM 5970. The 16S rRNA gene and pangenome phylogenetic trees (Figure 2A,2B) also demonstrated that CS13<sup>T</sup> and CP1 have the closest relationship. Therefore, we infer that the organism isolated from the blood of sheep on the Mongolian Plateau belongs to the genus *Carnobacterium* and that CS13<sup>T</sup> is on the same branch as *C. CP1*.

In contrast, the NR species distribution results indicated that CS13<sup>T</sup> had only 84.2% of protein coding genes that matched those of *C. CP1* (Figure 2C). Orthologous cluster analysis revealed that CS13<sup>T</sup> has 133 specific genes, in comparison with *C. CP1* (102 specific genes) and other related species (Figure 3A,3B), indicating that strain CS13<sup>T</sup> could be classified as a novel species of the genus *Carnobacterium*.

The protein sequence alignment of CS13<sup>T</sup> and *C. CP1* was carried out by the Circos package (Figure 4). The results illustrated that although the protein sequences of CS13<sup>T</sup> and *C. CP1* are also highly homologous, there are many specific fragments of CS13<sup>T</sup>, such as NODE\_88, NODE\_101, NODE\_113, NODE\_115 and NODE\_145, which further confirms CS13<sup>T</sup> as a novel species.

## Discussion

*Carnobacterium* is found mostly in the intestines of animals. As intestinal probiotics, certain *Carnobacterium* can also effectively inhibit pathogens and spoilage microorganisms, so they are widely used as a food additive. Among 12 species of *Carnobacterium*, *C. divergens* and *C. maltaromaticum* are frequently isolated from food, particularly in vacuum-packaged (VP) meat and meat products, which also show the ability to inhibit pathogenic and spoilage microorganisms

in diverse food matrices. Other scholars reported that *C. maltaromaticum* B26 and *C. divergens* B33, isolated from the intestine of healthy rainbow trout, are beneficial for enhancing cellular and humoral immune responses, and they were selected as potential probiotics with effectiveness. In this study, the *Carnobacterium antarticum* sp. CP1-like organism, CS13<sup>T</sup>, has been detected by PCR in the blood and in the intestinal contents of some sheep with diarrhea; however, the microorganism was sheep only isolated from the blood. This phenomenon is similar to animal diarrhea caused by conditional pathogens such as *Proteus mirabilis*, which can cause bacteremia through gastroenteritis [17]. However, the results of the physiological, biochemical and genetic analyses indicate that the microorganisms isolated from the Mongolian Plateau and Antarctica belong to the same novel species of the genus *Carnobacterium*. We suggest that this species is probably distributed globally and adapts to varying areas and surroundings. *Carnobacterium* species that have been isolated from the environment and are generally not characterized as pathogens, although they are known to cause disease in fish [18]. Only three reports describing *Carnobacterium* species from human infection have been found in the literature [19]. Here we report what we believe to be the first isolation and characterization of *Carnobacterium* species from bacteremia in sheep. Whether this novel species is related to animal disease or can be used in the food industry or other fields remain to be further studied.

## Funding Information

This work was funded by the Science and Technology Program of Chongqing Academy of Animal Sciences (19538), Chongqing, China.

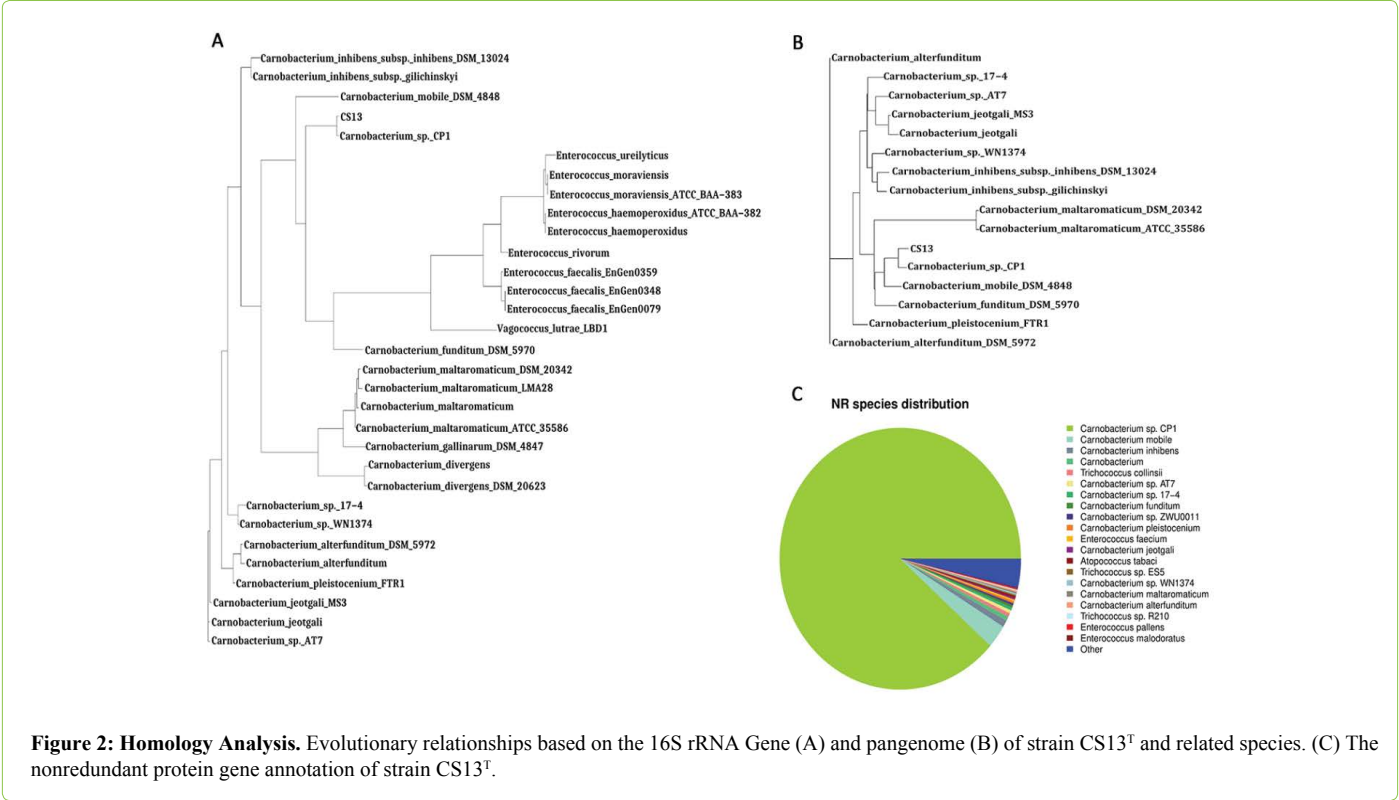
## Conflicts of Interest

The authors declare that there are no conflicts of interest.

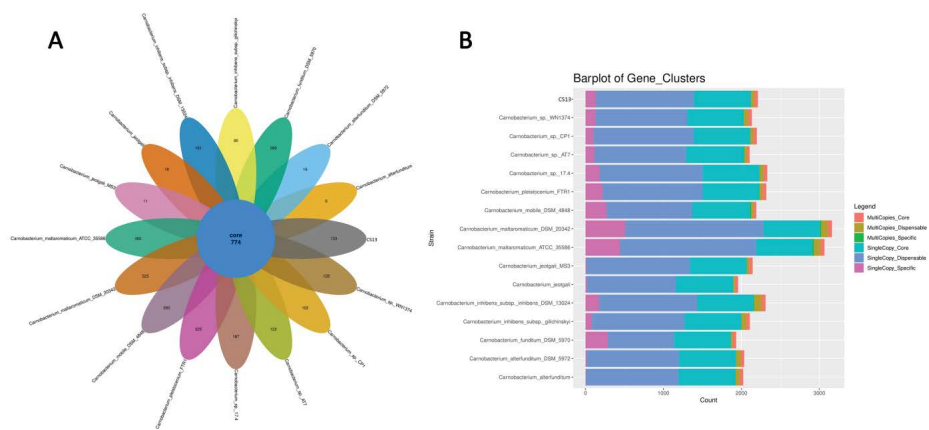
Characteristic	CS13 <sup>T</sup>	C. CPI <sup>*</sup>	C. DSM 4848 <sup>†</sup>	C. DSM 5970 <sup>‡</sup>
Isolation source	Sheep blood in Mongolian Plateau	Sandy soil in Antarctica	Frozen chicken meat	Lake water in Antarctica
Rods	+	+	+	+
Growth temperatures (°C) (optimum)	20-37 (30)	4-36 (28-32)	0-35	4-20 (4)
pH range (optimum)	5.0-9.0 (8.0)	6.0-9.5 (8.0-8.5)	ND	ND
NaCl tolerance range (w/v)	0-5.0 (1.0)	0-5.0 (1.0)	ND	1-2
Facultatively anaerobic	+	+	+	+
Hemolysis	+	+	ND	ND
Gram staining	+	+	+	+
Motility	+	+	+	+
Catalase	-	-	ND	-
Oxidase	-	-	ND	ND
Produce gas	-	-	+	ND
Produce H <sub>2</sub> S	-	-	-	ND
Acid from				
Cellobiose	+	-	+	-
D-Glucose	+	+	+	-
D-Mannose	W	+	+	-
Maltose	+	-	+	-
N-acetyl-glucosamine	+	+	+	-
Sucrose	+	+	+	-
Salicin	W	W	+	-
D-galactose	+	-	+	-
Arabitol	-	-	-	-
Glycogen	-	-	-	-
Starch	-	-	ND	-
Xylitol	-	-	-	-
Glycerol	W	W	ND	-
Esculin	W	W	ND	+
DNA G+C content (%)	37.84	38.1	35.5-37.2	34

\* Data for *C. antarcticum* were taken from Zhu et al. [2].  
† Data for *C. mobile* were taken from Collins et al. [15].  
‡ Data for *C. iners* were taken from Snauwaert et al. [5].

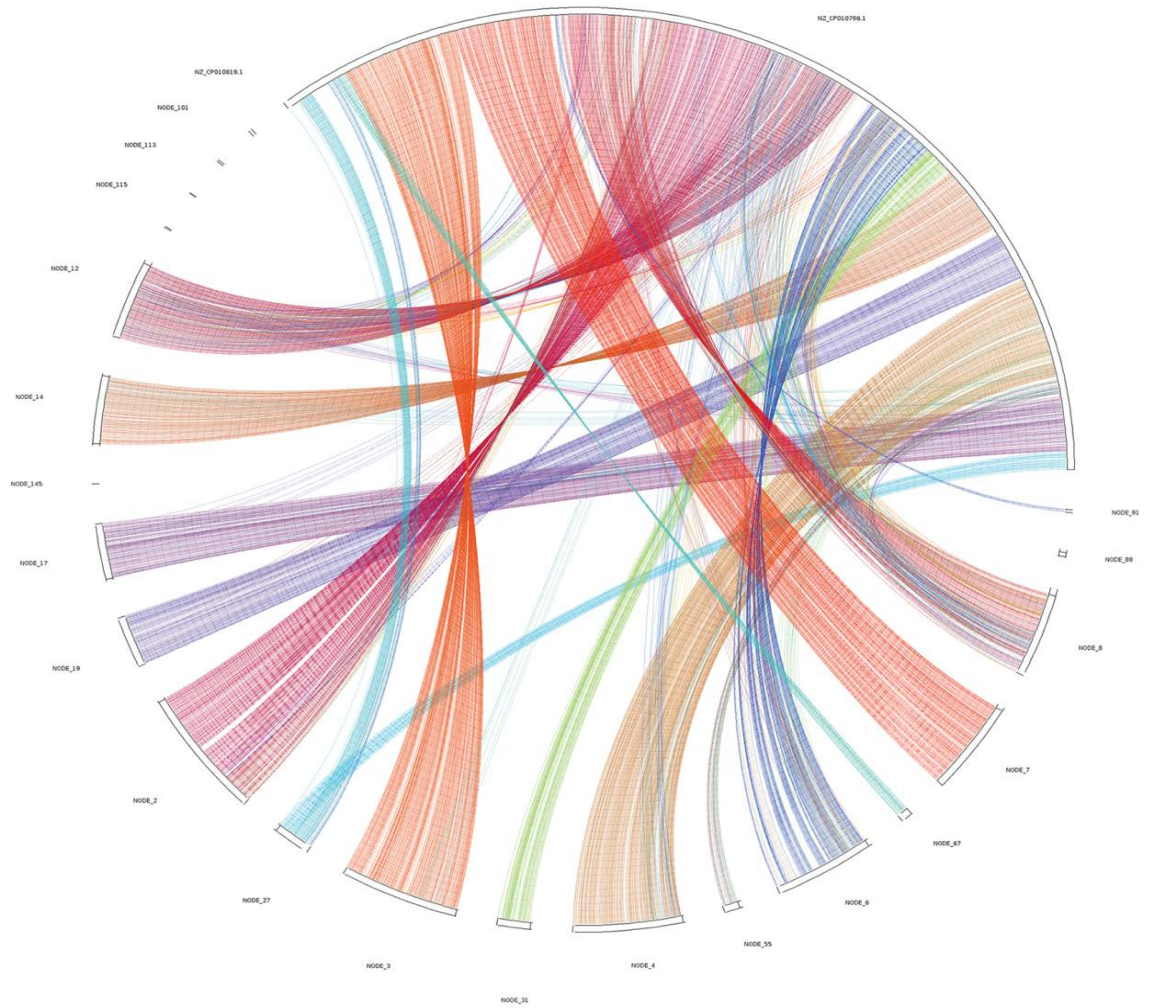
**Table 1:** Differential Physiological Characteristics Distinguishing Strain CS13<sup>T</sup> from the Closest Members of the Genus *Carnobacterium*. +, -, W and ND Represent Positive, Negative, Weakly Positive and ‘No Data’, Respectively.







**Figure 3: Pan Genomic Analysis.** (A). Quantity of core genes (center) and specific genes (petal) of strain CS13<sup>T</sup> and related species. (B). Gene clusters of strain CS13<sup>T</sup> and related species.



**Figure 3: Pan Genomic Analysis.** (A). Quantity of core genes (center) and specific genes (petal) of strain CS13<sup>T</sup> and related species. (B). Gene clusters of strain CS13<sup>T</sup> and related species.

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