

Biochemical and Molecular Characterization of a Native Haloalkalophilic Tolerant Strain from the Texcoco Lake

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Abstract

In the last decade several new genera have been isolated in alkaline and halophile growth conditions. The studies conducted in the Texcoco Lake soils have shown a generalized microbial adaptation to the specific conditions. In this research work, morphological and phylogenetic characterization of the HN31(22) strain that was isolated from the cited soil is presented. The strain was identified as a Gram-positive halophile and alkaline tolerant bacteria from the *Nesterenkonia* genus, which uses different substrates in metabolic processes.

Key words: halophile, alkalophile, adaptation, phylogeny, *Nesterenkonia*

Introduction

Alkalophile microorganisms are defined as organisms that have an optimum growth rate in environments with alkaline pH, particularly greater than 8. A source rich in variety of these organisms are the environments with a stable alkalinity, such as saline lakes (Castillo et al. 2005). Halophile bacteria are defined as microorganisms that show a better growth in the presence of salt NaCl. Given that these microorganisms are capable of growth in different saline concentrations, the term halophile is usually reserved for those requiring a minimum of excess salt concentration, found in seawater, in general, concentrations greater than 3% (Oren, 2008). Extreme halophile bacteria are those that present an optimum growth rate in high saline concentrations, near 20% NaCl (3–4 M). These bacteria grow in hyper saline environments, such as saline lakes that present a greater range of saline concentrations than sea (Jones and Grant 2002). The soil from the Texcoco Lake is alkaline, and with electrical conductivities (EC) in saturated strata from 22 to 150 dSm⁻¹ and sodium percentages from 76% to 98% (Luna-Guido et al. 2002). Several microorganisms adapted to extreme conditions have been identified from the soil of the former Texcoco

Lake (Valenzuela-Encinas et al. 2008; Ruiz-Romero et al. 2013; Soto-Padilla et al. 2014). The application of molecular biological techniques to microbial ecology has shown that the cultured organisms are generally different from those that dominate in the natural environment (Oren, 2002). In addition, knowledge of their biochemical characteristics supports understanding of their metabolic processes.

In the last decade, there has been an increment in the number of newly isolated bacterial genera, which grows in alkaline and halophile conditions. The study of enzymes that aid in the metabolism of these extremophiles to operate under these conditions is of great interest (Castillo et al. 2005; Ramírez et al. 2006). Characterization and identification of native microorganisms of relevant sites, as well as specific biological and metabolic functions are closely related to protein and enzymes that work in extreme conditions. These biomolecules show unique features that can be used as models for the design and construction of proteins with new properties, which are of interest for industrial applications (Castillo et al. 2005). The aim of this study was to accomplish biochemical and phylogenetic characterization of the native halophile/alkaline tolerant bacteria from the Texcoco Lake.

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Experimental

Materials and Methods

Microorganisms. The bacterial strain HN31(22) was isolated from the soil of the Texcoco Lake, located northwest from Mexico city (Northern Latitude 19°30'52' Western Longitude 98°59'24') in the State of Mexico, Mexico (Soto-Padilla et al. 2014). The strain was cultivated and maintained in marine agar (Bacto marine agar 2216, DIFCO). Growth conditions were as described by Valenzuela-Encinas et al. (2008). Pure cultures were obtained by picking and restreaking individual colonies grown in Petri plates solidified with 2% agar at a temperature of 37°C for 48 h.

Tolerance to pH, temperature and NaCl concentration. A commercial marine medium (Bacto marine broth 2216, DIFCO) was used. The pH values from 4 to 12 were tested at a temperature of 37°C, and NaCl percentage concentrations of 1.9, 5, 10, 15 and 20 were employed. In order to determine the growth rate curves, the turbidity measurement (Cintra 10e, GBC Scientific Equipment, Australia) at 600 nm (Coronado et al. 2000; Thacker et al. 2006) to subsequently determine cell concentration using the dry biomass technique was performed. The dry weight of the bacterial cells was obtained after centrifugation of a known volume of the culture suspension and drying the pellet in an oven at 105°C for 24 hours. To establish the optimum growth temperature, the strain was incubated at various temperatures of 4, 30, 37, 40, 55°C. All experiments were done in triplicate.

16S rRNA gene sequencing and phylogenetic analysis. DNA extraction was done for phylogenetic characterization, including amplification, sequencing and bioinformatics analysis (NCBI) (Soto-Padilla et al. 2014). The 16S rRNA gene was amplified using the universal bacterial forward and reverse primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), respectively. The reaction mixture (25 µl) contained 1 µl of genomic DNA; the appropriate primers (27F and 1,492R) at 0.5 µM each one; dATP, dCTP, dGTP, and dTT at 10 mM each one; 50 mM MgCl₂; and 1 U of Taq DNA polymerase in the PCR buffer provided by the manufacturer (Invitrogen, USA). Amplification conditions included: denaturation of the sample for 10 min at 94°C, followed by 1 min of annealing at 57°C and elongation during 2 min at 72°C for a total number of 35 cycles (Valenzuela-Encinas et al. 2008). The amplification was performed in a Touchgene Gradient thermal cycler FTGRAD2D (TECHNE DUXFORT, Cambridge, UK). Phylogenetic trees were constructed using the Neighbor-joining method; and Tamura-Nei model of distance analysis and 500 Bootstrap replications were assessed to support internal branches.

Morphologic and biochemical characterization.

To determine the bacterial cell morphology, the scanning electron microscopy (SEM) was used. The cells from a 24 hours culture were collected, washed, fixed with 2% formaldehyde, and dried by dehydration using acetone and ethyl alcohol. The following biochemical tests were performed: Gram staining, oxidase activity, catalase production, urease, nitrate reduction, citrate test, MR-VP, oxidation and fermentation of sugars. Utilization of glucose, trehalose, mannitol, sucrose and fructose were evaluated according to Cowan and Steels methodology (Barrow and Feltham, 2004). Hydrolysis of Tween 80, casein, gelatin and starch were also performed using standard techniques. All experiments were done in triplicate.

Results

The HN31(22) strain was Gram-positive with cells of short rods morphology (Fig. 1) and size between 1–2 µm. Colonies showed a light yellow coloration, with bright creamy and round shape and flat borders. Growth of the bacteria was observed in NaCl at concentrations of up to 20% of (Fig. 2); the most efficient growth rate was observed when 5% NaCl was used, but the bacteria grew also when 10, 15 and up to 20% salt concentrations were employed. With this evidence, the bacterium was considered a halophile. Fig. 3 shows the growth of HN31(22) strain in the evaluated pH values, showing that an optimum growth rate was obtained at a neutral pH of 7. The bacterial multiplication was also observed at pH values of 5 to 10; however, a strong reduction was registered at pH of 11. For this reason the strain was considered as an alkaline tolerant bacteria. Table I shows the growth of the HN31(22) strain in various temperatures.

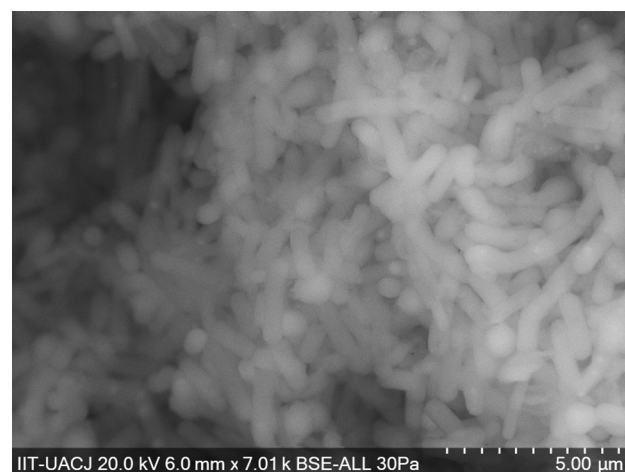


Fig. 1. A high resolution image of bacterial colonies of the HN31(22) strain taken under scanning electron microscope (SEM).

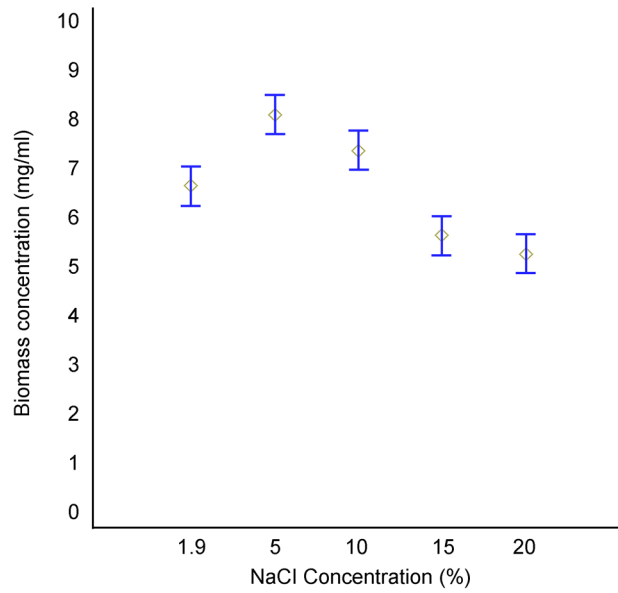


Fig. 2. The concentration of biomass (mg/ml) of the HN31(22) strain in marine medium at different NaCl concentrations (1.9–20%).

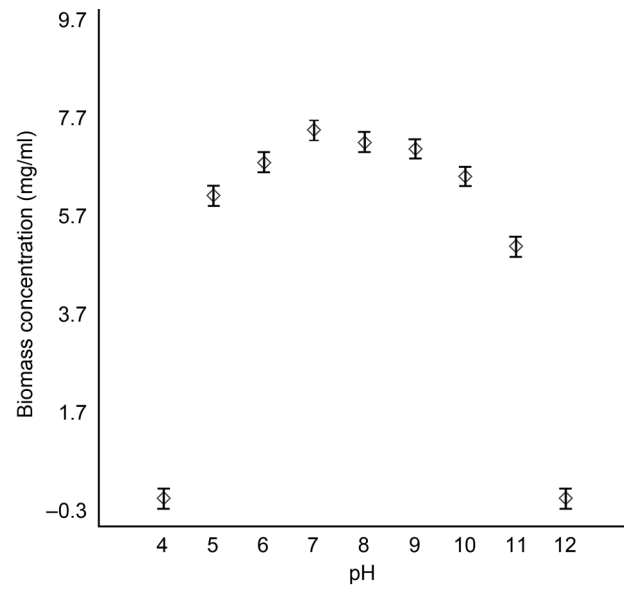


Fig. 3. The concentration of biomass (mg/ml) of the HN31(22) strain in marine medium at different pH (4–12).

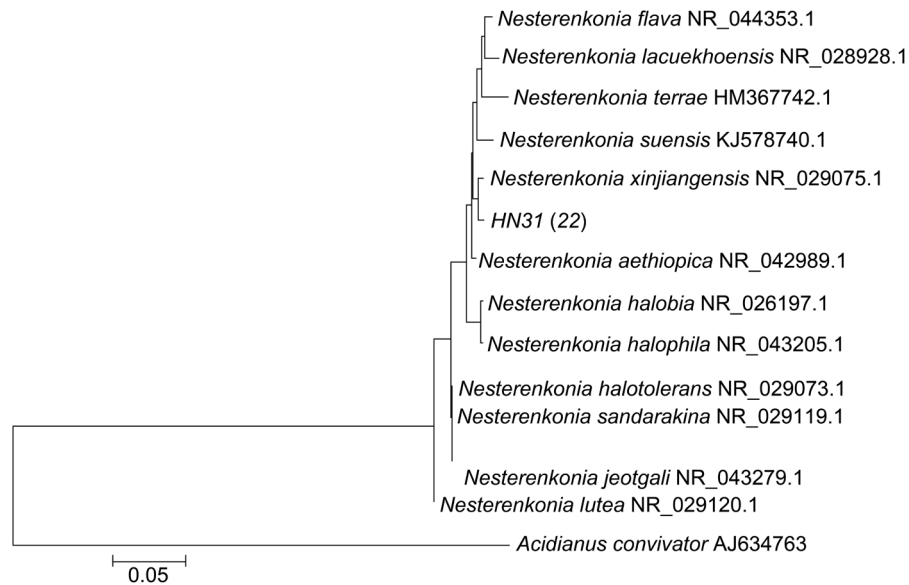


Fig. 4. Phylogenetic relationship of species of the *Nesterenkonia* genus based on the 16S rDNA gene sequences.

A phylogenetic analysis was carried out and the strain isolated was classified to the genus of *Nesterenkonia* (Fig. 4) with features and shared values between

Table I
Growth of bacteria of the HN31(22) strain in marine agar at different temperatures (4, 30, 37, 40, 55°C).

Temperature (°C)	Growth (CFU/ml)
4	0
30	4.2×10^3
37	34×10^4
40	18×10^4
55	0

97.1% and 99.5% with other species within this genus. The evaluated biochemical traits show common characteristics with the data reported on the *Nesterenkonia* genus (Table II), in which the capacity of HN31(22) strain to use several substrates and perform different hydrolytic activities (amylase, protease, lipase) was underlined.

Discussion

Research studies performed on the microorganisms of the Texcoco Lake, showed that there has been a great microbial adaptation to the extreme

Table II

Comparison of phenotypic characteristics of bacteria of the HN31(22) strain with the other species of *Nesterenkonia* (1. *N. flava*, 2. *N. lacusekhoensis*, 3. *N. suensis*, 4. *N. xinjiangensis*, 5. *N. halotolerans*, 6. *N. aethiopica*, 7. *N. sandarakina*, 8. *N. lutea*, 9. *N. jeotgali*, 10. *N. halophila* (Collins et al. 2002; Li et al. 2004, 2005, 2008; Delgado et al. 2006; Yoon et al. 2006; Luo et al. 2008; Govender et al. 2013).

Characteristic	HN31(22)	1	2	3	4	5	6	7	8	9	10
Morphology	Short roads	Short roads	Short roads	Short roads	Short roads	Cocci	Short roads	Cocci	Cocci	Cocci	Cocci
Tincion	HN31(22)	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +
Colony pigmentation	Yellow	Yellow	Yellow	Yellow	Yellow	Orange/Yellow	Yellow	Orange/Yellow	Yellow	Yellow	White ivory
Temperature (°C)	30–40	40–42	8.5–42	35–37	20–40	4–40	25–40	4–36	4–36	4–36	NR
pH	5–11	8–12	7.5–9.5	7–11	7–12	7–9	7–11	5–12	6.5–10	6–8.5	6–10.5
NaCl (%)	0–20	0–10	0–15	0–18	0–25	0–25	3–12	1–15	0–20	0–16	0.5–30
Catalase	+	+	NR	+	NR	NR	+	NR	NR	NR	NR
Oxidase	–	–	–	–	–	–	+	–	–	–	–
Urease	–	NR	NR	NR	+	+	–	–	–	–	NR
Nitrate reduction	–	–	–	+	–	–	NR	–	+	–	+
Citrate test	+	–	W	–	NR	NR	–	NR	NR	NR	NR
Voges-Proskauer	–	–	–	–	NR	NR	–	–	–	+	NR
Acid produced from:											
Galactose	–	–	–	–	–	–	–	+	+	+	NR
Trehalose	+	–	+	–	–	–	–	W	+	W	–
Mannitol	+	–	–	–	–	–	–	+	+	+	NR
Sucrose	+	NR	NR	–	–	–	–	NR	NR	NR	–
Fructose	+	NR	NR	+	–	–	+	NR	NR	NR	NR
Utilization of:											
Glucose	+	+	NR	+	+	+	+	+	W	+	+
Trehalose	+	–	+	–	–	–	–	+	–	+	NR
Fructose	–	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	–	+	+	W	+
Sucrose	+	+	NR	+	+	+	W	+	+	+	NR
Maltose	+	+	NR	–	+	+	NR	+	+	+	NR
Hydrolysis of:											
Starch	+	+	–	+	–	–	+	–	+	–	–
Gelatin	+	+	–	NR	+	+	+	+	–	–	NR
Tween 80	+	+	–	+	NR	NR	NR	–	–	–	–
Casein	+	NR	NR	–	+	–	+	–	+	NR	–

(+) Positive, (–) negative, (w) weak reaction, (NR) unreported.

environmental conditions (Luna-Guido and Dendoven 2001; Soto-Padilla et al. 2014; Valenzuela-Encinas et al. 2008). Soto-Padilla et al. (2014) reported the presence of the genera with halophile and alkaline characteristics, such as: *Salinicoccus*, *Kocuria*, *Micrococcus* and *Nesterenkonia*. Morphologic characterization has been done for a new strain of *Nesterenkonia* genus, and the existing relation with the bacteria reported (Table II) concurs with those of Gram-positive bacteria, yellow colonies, and growth at pH values of alkaline range, and in high salt concentrations (NaCl). Ramírez et al. (2006) proposed bacterial classification as a function of the required salt needed for growth; and based on the

data from Fig. 2 and Table II, the HN31(22) strain has been classified as a halophile. The applications of this type of bacteria in biotechnology can be divided into a number of categories; for example, food, plastics and pharmaceutical industry and bioremediation (Castillo-Carvajal and Barragán-Huerta 2011). The halophile tolerance of enzymes can be exploited when enzymatic activities are required in low water activity, such as those found in the presence of high salt concentrations. The interesting product applications for some organic osmotic stabilizers, produced by halophiles, have also been found. Several halophile microorganisms produce valuable compounds (enzymes, biopolymers), some of

those are unique and cannot be found in other type of microorganism (Oren 2002). Oarga (2009) defined an alkaline microorganism, as one with an optimum growth rate above two units above the neutral pH, and that requires high pH values for its growth; each organism has a defined pH interval, in which growth is possible (optimum pH). Most microorganism grow at a pH range between 5–9, only a few species can grow in pH values above 10 and below 2. It has been known that very few microorganisms are capable to grow in pH values close to 0 (Castillo et al. 2005).

The *Nesterenkonia* genus belongs to the Micrococcaceae family; it was proposed by Stackebrandt et al. (1995), who performed taxonomic dissection of the genus *Micrococcus*. Initially only the *Nesterenkonia halobia*, originally classified as *Micrococcus halobius* (Onishi and Kamekura 1972), isolated from unrefined salt from Noda in Japan was included in this genus. There has been already 14 families reported, all of which represent extremophile microorganism species (Bakhtiar et al. 2003; Bakhtiar et al. 2005; Amoozegar et al. 2007). Species belonging to the *Nesterenkonia* genus have been applied for the production of certain enzymes and metabolites of interest given their biochemical characteristics (Rivadeneira et al. 2000; Govender et al. 2009; Shafiei et al. 2010, 2012; Nel et al. 2011). The HN31(22) strain isolated from the Texcoco Lake, was classified as a Gram-positive, halophile/alkaline tolerant microorganism from the *Nesterenkonia* genus that uses starch, gelatin, casein and Tween 80 as its metabolic substrates.

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