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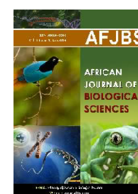

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Description of *Nitrincola elmenteitensis* sp. nov.; a haloalkaliphilic bacterium isolated from Lake Elmenteita in Kenya

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Abstract

A bacterial strain designated E-48^T was isolated from Lake Elmenteita, an alkaline saline lake within the East African rift valley. The cells were Gram-negative, motile, non-spore forming rods. Growth was observed at a pH range between 6.0 and 13.0 (optimum pH 9.5), salt concentration (w/v) up to 20% (optimum 5%) and the temperature for growth was between 14.0-44 °C (optimum temperature 36 °C). On the basis of 16S rRNA gene sequence similarity (99%), strain E-48^T belonged to the genus *Nitrincola* (family *Oceanospirillaceae*, class *Gammaproteobacteria*). The DNA G-C content was 46.4 mol%. The major cellular fatty acid was the mono-unsaturated 18:1 ω7c while the major isoprenoid quinone was Q-8. However, at the genome level, strain E-48^T had a G+C difference of 7.17% from the genome of *N. lacisaponensis* DSM 16316^T. Based on the various characteristics, it is proposed that the isolate represents a new species within the *genus Nitrincola* for which the name *Nitrincola elmenteitensis* is proposed. The type strain is E-48^T (=DSM 26266^T=LMG 28382^T).

Keywords: *Nitrincola, Gamaproteobacteria, Soda Lakes*

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1. Introduction

The genus *Nitrincola* (Dimitriu *et al.*, 2005) in the family of *Oceanospirillaceae* is currently comprised of five validly published species. Most of the genera in this family are either halophiles or halotolerant. *Nitrincola lacisaponensis* is the type species of this genus and was isolated from decayed wood collected at a meromictic alkaline saline lake in the USA (Dimitriu *et al.*, 2005). *N. alkalisediminis* was isolated from the alkaline Lonar Lake in Maharashtra, India (Joshi, A. *et al.*, 2016) while *Nitrincola schmidtii* and *Nitrincola alkalilacustris* (Borsodi *et al.*, 2017) were recovered from soda pans located in the Kiskunság National Park, Hungary. More recently, *Nitrincola tibetensis* xg18^T was isolated from Lake XuguoCo on the Tibetan Plateau (Phurbu *et al.*, 2019). *Nitrincola nitratireducens* AK23^T is an effectively but not validly published species isolated from Lonar Lake, Buldhana district, India (Singh *et al.*, 2015). In this study, we describe a novel E-48^T isolated from the haloalkaline Lake Elmenteita in Kenya.

2. Materials and methods

Serially diluted sediment samples were plated on a medium containing 0.02% starch, 0.01% yeast extract, 0.01% peptone, 1.5% agar and cycloheximide (100 mg/L) to inhibit the growth of fungi. This medium was

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prepared using filter sterilized autoclaved water collected from Lake Elmenteita. A pure culture was obtained by repeated streaking on modified tryptic soy broth (supplemented with 15 g Bacto agar (Difco), 3.5% NaCl and 1% Na_2CO_3). The pure isolates were stocked in tryptic soy broth supplemented with 3.5% NaCl, 1% Na_2CO_3 and 20% (v/v) glycerol. *Nitrincola lacisaponensis* DSM 16316^T from the DSMZ was used as the reference strain and grown under the same laboratory conditions.

Colony morphology was observed on cultures grown on the modified tryptic broth for 24-48 h using a stereo-microscope. Gram staining was done as described (Claus DA, 1992) to observe the shape, size and arrangement of the cells. Catalase activity was tested using H_2O_2 test as described (Zimmermann *et al.*, 1990). The following physiological and biochemical properties were examined: oxidation/fermentation of glucose; arginine dihydrolase; tyrosine decarboxylase, colony pigmentation, cell morphology, ability to hydrolyze gelatine, DNase activity, starch utilization and Tween-80. Chitinase activity was tested by use of 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide. Motility was tested with cells from 2-day-old liquid cultures on soft agar (0.4%) incubated for 48 h at 28 °C. Cytochrome C oxidase was determined by adding a few drops of tetramethyl-phenylenediamine solution to a 2-day-old slant of each strain.

Utilization of glucose, sucrose, fructose, lactose and mannitol as sole carbon sources was tested on a basal media containing per liter 1 g yeast extract (Difco), 1g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.05 g $CaCl.2H_2O$, 4% NaCl and 1% Na_2CO_3 and the respective sugar to a final concentration of 1%. Growth was measured after 72 h at 28 °C using a spectrophotometer (turbidimetry) at a wavelength of 600 nm. Salt tolerance tests were done on diluted nutrient broth (Difco) supplemented with 1% Na_2CO_3 and the salt concentration was varied from 0% to 20%. The optimum temperature for growth was determined on TSB containing 4% (w/v) NaCl and 1% Na_2CO_3 using a temperature gradient incubator model TN-3 (Toyo Kagaku Sangyo) with the lowest value of 9.4 °C and the highest 49.3 °C. The optical density was recorded after 18 h of growth using a spectrophotometer at 620 nm. The pH range for growth (6.0 to 13) was determined in nutrient broth diluted 10 times with the pH adjusted using phosphate buffer and a salt concentration of 4%. The ability to oxidize or utilize organic substrates was investigated using BIOLOG-GN plates as recommended by the manufacturer. The results were read using the BIOLOG microplate reader after incubation for 6 h, 24 h, 48 h, and 72 h and after five days of growth. Carbon assimilation tests were also determined using the commercial API 20E and API 50 CH systems (BioMe´rieux). Tests were read after 6 h, 24 h, 48 h, and 72 h and after five days of growth.

Cell biomass for fatty acids, isoprenoid quinone and polar lipids analyses was obtained by cultivation on tryptic soy broth supplemented with 3.5% NaCl and 1% $NaCO_3$ at pH 9 and 28 °C while shaking at 150 rpm for 24 h. These conditions were chosen based on the fact that optimum growth occurred at 4% NaCl (tryptic soy broth already has 0.5g/L) and pH of 9. The cells were thereafter harvested during the mid-exponential growth phase and freeze-dried. Cells for electromicrography were grown the same way but processed after 24 h. Cellular fatty acids were extracted as described (Stead *et al.*, 1992) and analyzed on an Agilent 6890N gas chromatography system. The data generated was analyzed for taxonomic information by the TSBA40 and TSBA50 method of the Sherlock MIS software. The individual fatty acids were expressed as percentages of the total fatty acids. Respiratory lipoquinones and polar lipids were extracted and analyzed as described (Tindall, 1990a and 1990b; and Altenburgera *et al.*, 1996). DNA extraction, PCR amplification and sequencing of the 16S rRNA genes were performed at SeqLab (Göttingen, Germany). Identification of phylogenetic neighbors and calculation of pairwise 16S rRNA gene sequence similarity was done using the EZBioCloud e-server (https://www.ezbiocloud.net (Yoon *et al.*, 2017). Phylogenetic relationship was determined using neighbor-joining (Saitou and Nei, 1987; and Felsenstein, 1993) and maximum-likelihood analyses (Olsen *et al.*, 1994). These analyses were conducted in MEGA 7 (Tamura *et al.*, 2011). The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004). The resultant tree topologies were evaluated in bootstrap analyses of the neighbor joining method based on 1000 resamplings (Felsenstein, 1985). The16S rDNA sequence (1,445bp) was deposited under the accession number FJ764762. Cells for DNA base composition were grown as described earlier, harvested and disrupted by using a Constant Systems TS 0.75 kW (IUL Instruments). DNA in the crude lysate was purified by chromatography on hydroxyapatite as described (Cashion *et al.*, 1977). The mol% G + C content of the DNA was determined by reversed-phase HPLC of nucleosides as described (Mesbah *et al.*, 2007). DNA-DNA hybridizations between the genome sequence of strain E48^T and its phylogenetic neighbor *Nitrincola lacisaponensis* DSM 21637^T were done based on *in silico* genome-to-genome comparisons (GGDC) (http://ggdc.dsmz.de) to test whether the new strain was novel using the genome of strain *Nitrincola elmenteitensis* E48^T and *Nitrincola lacisaponensis* DSM 21637^T as described (Meier-Kolthoff *et al.*, 2014 and 2013).

3. Results

Strain E48^T formed circular, entire, smooth yellowish colonies on the modified Trypticase soy broth medium after 24-48 h of incubation at 37 °C. Cells were Gram-negative, aerobic, oxidase and catalase positive non-spore forming rods with a monopolar flagellum. The cell morphology is shown (Figure 1).

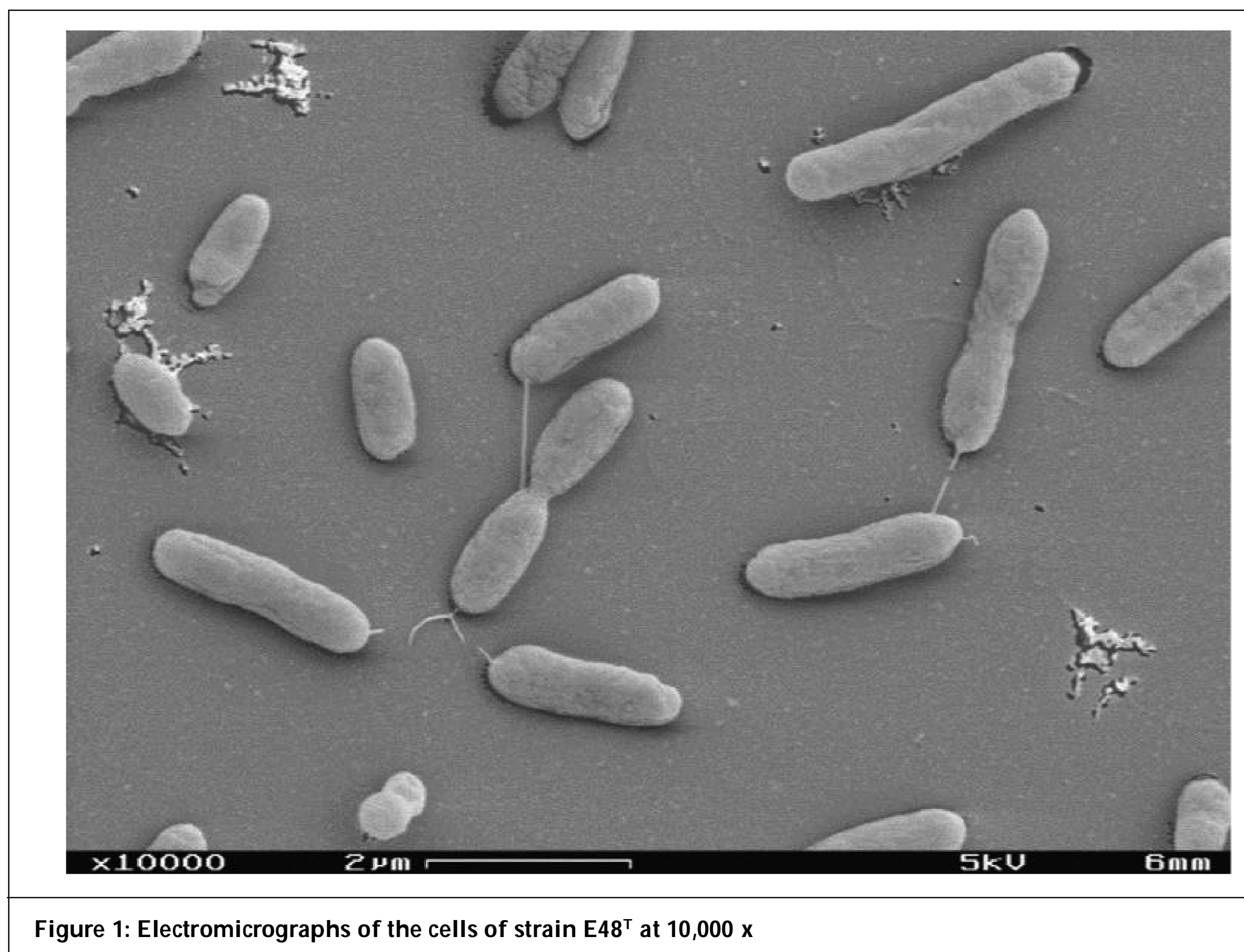

Figure 1: Electromicrographs of the cells of strain E48^T at 10,000 x

The physiological characteristics of strain E-48^T and other closely-related type strains of species of the genus *Nitrincola* are presented in Tables 1a and b. Notable is that strain E48^T and *N. lacisaponensis* had almost

Table 1a: Physiological characteristics of the Isolates: 1, E-48^T; 2, *Nitrincola lacisaponensins* (DSM 16316^T) (This study); 3, *N. alkalisediminis* JCM 19317^T (Joshi *et al.*, 2016) 4, *N. nitratireducens* JCM 18788 (Singh et al., 2015), *Nitrincola alkalilacustris* strain ZV-19^T and *Nitrincola schmidtii* strain R4-8^T (Borsodi *et al.*, 2017)

	1	2	3	4	5	6
pH Range	6.0-11.5	6.0-11.0	7-11	7-11	7-11	7-11
Optimum pH	7.5-9.5	9.5	8-10	8-10	8-9	8-9
NaCl range (w/v, %)	0-16	0.6-12.0	0-7	0-7	0-10	0-10
NaCl optimum (w/v, %)	5	8	0-5	0-5	0-7	0-7
Temperature Range (°C)	22.0-44.0	22.3-46.1	15-40	10-40	10-37	10-28
Temperature Optimum (°C)	36.0	34.7	25-28	25-28	20-28	15-20
*G+C Content	46.4	52.1	49.3	46.8	54.5	45.8

Note: * The G+C values for the comparative strains are derived from the NCBI genomes database.

similar temperature, pH and salinity range of growth which was different from the other strains in the genus. This is probably a reflection of the soda lake habitats from which the two strains were isolated from.

Table 1b: Biochemical characteristics of the Isolates E-48^T and *Nitrincola lacisaponensis* (DSM 16316^T) (This study)

	E-48^T	*Nitrincola lacisaponensis*
Acetic Acid	–	+
Dextrin	+	+
L-Threonine	–	+
α-D-Glucose- 1-Phosphate	–	+
D-Glucose- 6-Phosphate	–	+
Arbutin	+	+
Esculin/ferric citrate	+	+
D-Saccharose (sucrose)	+	–
Glycogen	–	+
D-Turanose	+	+
Propionate	–	–
Succinate	–	+
Potassium 5-KetoGluconate	+	+
Indole	w	–
VP	–	–
Tween 40	+	–
Nitrate reduction		

Ubiquinone (Q-8) was the major respiratory quinone (at 95.2%) detected in strain E-48^T while the cellular fatty acid profiles were dominated by $C_{18:1\,\omega7c}$, $C_{16:1\,\omega7c}$ and $C_{16:0}$. This concurs with the results reported so far in other species of the genus *Nitrincola* (Table 2).

The G+C content of the genomic DNA was 46.68% which is lower than the other described species in the genus (Table 1a). However, DNA-DNA comparison results show a G+C difference of 7.17% and therefore delineates E48^T as a distinct species from *N. lacisaponensis* 4CA^T (=DSM 16316^T). The major polar lipids in strain E-48^T were phosphatidylglycerol and phosphatidylethanolamine and minimal amounts of Diphosphatidylglycerol. However, in *Nitrincola lacisaponensis*, Diphosphatidylglycerol was present in significant amounts (Figure 2). This is another feature that distinguishes strain E-48^T from *N. lacisaponensis.* The major polar lipids in strain E-48^T were phosphatidylglycerol and phosphatidylethanolamine while the major fatty acids were C10:0, C10:0 3OH, C12:0, C16:1 ω7c/15 ISO 2OH, C16:0 and C18:1 ω7c.

Based on EzBioCloud (Yoon *et al.*, 2017), the most closely related type strain to E48 was *N. lacisaponensis* 4CA^T (=DSM 16316^T) showing 98.81% pairwise 16S rRNA gene sequence similarity value with 16 mismatches. Phylogenetic analysis using the 16S rRNA gene confirms that E48^T belongs to the genus *Nitrincola* (Figure 3).

4. Description of *Nitrincola elmenteitensis* sp. nov.

El.men.teit.en'sis. N.L. masc. adj. elmenteitensis, of or pertaining to Lake Elmenteita in Kenya where the strain was isolated.

The bacterial strain designated E-48[T] was isolated from Lake Elmenteita, an alkaline saline lake within the East African rift valley. Cells are gram-negative, aerobic, oxidase and catalase positive and non-spore forming rods. The cells are about 2 μm in length and occur singly or in pairs. Motility is via a monopolar flagellum and

Table 2: Fatty acid composition of the isolates: 1, E-48[T]; 2, *Nitrincola lacisaponensins* (DSM 16316[T]) (This study); 3, *N. alkalisediminis* JCM 19317[T] (Joshi *et al.*, 2016) 4, *N. nitratireducens* JCM 18788[T] (Singh et al., 2015), 5, *Nitrincola alkalilacustris* strain ZV-19[T] and 6, *Nitrincola schmidtii* strain R4-8[T] (Borsodi *et al.*, 2017)

Fatty Acid	1	2	3*	4*	5*	6*
$C_{10:0}$	2.04	3.53	3.5	1.9	TR	3.1
$C_{10:0\ 3OH}$	2.75	4.76	5.3	4.0	2.8	5.4
$C_{12:0}$	2.17	3.31	3.7	2.1	1.9	TR
$C_{16:1\ \omega7c/15\ ISO\ 2OH}$	23.95	17.45	30.5	24.4	19.1	22.7
$C_{16:0}$	19.22	15.11	10.2	12.8	10.1	13.4
$C_{18:1\ \omega7c}$	48.68	53.38	46.5	53.4	63.0	50.9

Note: * Data from Barsodi *et al.*, 2017.

the strain grows at pH between 6.0-11.5. The optimum pH for growth is between 7.5 and 9.5. Though growth occurs at a salt concentration ranging between 0 and 16%, the optimum concentration is 5%. Temperature for growth ranges from 22-44 °C and an optimum of 36 °C. Dextrin and Tween 80 are hydrolyzed on BIOLOG GN test results and is negative for all the other substrates. D-xylose, D-mannose, L-sorbose, D-sorbitol, arbutin, esculin/ferric citrate, D-turanose, potassium 5-ketogluconate are positive on the API 50CH. Nitrate reductase is absent. No pigment is produced after prolonged growth. The major polar lipids in strain E-48[T] are phosphatidylglycerol and phosphatidylethanolamine while the major cellular fatty acids are C10:0, C10:0 3OH, C12:0, C16:1 ω7c/15 ISO 2OH, C16:0 and C18:1 ω7c. The 14:0 iso-branched cellular fatty acids are

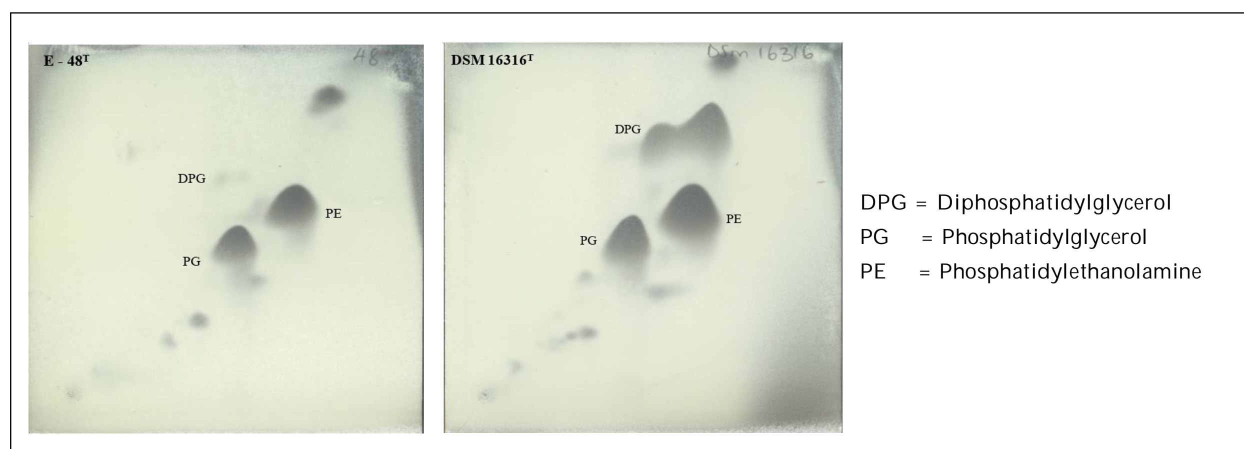

Figure 2: Polar lipid profiles of strain strain E-48[T] (*Nitrincola elmenteitensis* sp. nov.) and *Nitrincola lacisaponensins* DSM 16316[T] after separation by two-dimensional TLC as described (Tindall 1990a; 1990b; Altenburgera et al., 1996)

absent. Features that differentiate strains of *Nitrincola elmenteitensis* sp. nov. from the other strains is the ability to utilize D-xylose, D-mannose, L-sorbose and D-sorbitol. The DNA G-C content is 48.68 mol% while the G+C content difference (based on the in silico DDH method) between the two genomes (E-48[T] and *N. lacisaponensis* 4CA[T]) is 7.17%. Based on the various characteristics, it is proposed that the isolate represents a new species within the *genus Nitrincola* for which the name *Nitrincola elmenteitensis* is proposed. The type strain is E-48[T] (=DSM 26266[T]=LMG 28382[T]) with the 16S rRNA accession number FJ764762. The WGS accession number is SAMN03247511.

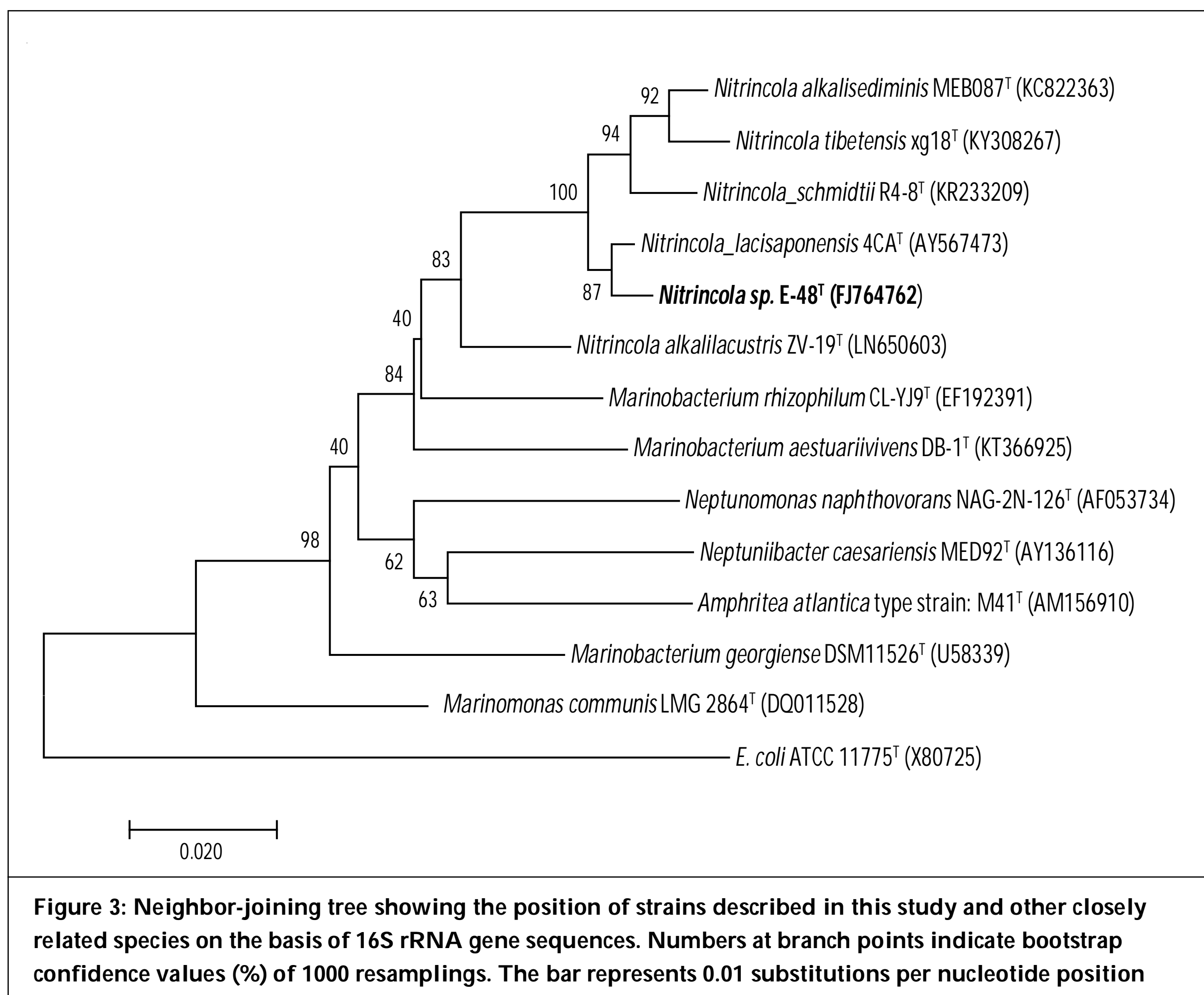

Figure 3: Neighbor-joining tree showing the position of strains described in this study and other closely related species on the basis of 16S rRNA gene sequences. Numbers at branch points indicate bootstrap confidence values (%) of 1000 resamplings. The bar represents 0.01 substitutions per nucleotide position

5. Conclusion

Soda lake ecosystems are characterized by high microbial diversity comparable to that of soil habitats. However, these habitats pose a challenge to most living systems due to unique physicochemical gradients. For example, organisms that thrive in the haloalkaline ecosystems have adapted to the extreme conditions of high pH, elevated salt concentrations and at some locations elevated temperatures. Therefore, the ability of isolate E-48 T to grow at pH up to 13 and optimum salt concentration of 5% (w/v) reflects adaptation to the habitat from which it was isolated. Based on the various characteristics, we conclude that the isolate represents a new haloalkaliphilic species within the genus Nitrincola for which the name Nitrincola elmenteitensis is proposed.

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