Nitrincola lacisaponensis gen. nov., sp. nov., a novel alkaliphilic bacterium isolated from an alkaline, saline lake

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A novel alkaliphilic bacterium, strain 4CA^T, was isolated from decomposing wood taken from the shore of Soap Lake, a saline, alkaline lake in Grant County, WA, USA. Cells of the isolate were Gram-negative, asporogenous, short, motile rods that utilized only a limited range of organic acids as sole carbon and energy sources. In addition to oxygen, the strain possessed the ability to reduce NO₂⁻ in the presence of acetate. Strain 4CA^T was oxidase- and catalase-positive; it degraded Tween 60, but not DNA, urea, gelatin or starch. It grew at pH values from 7.5 to 11.0, with optimum growth occurring at pH 9.0, and growth was observed in NaCl concentrations of 0.2–1.3 M, with optimum growth at 0.8 M NaCl. The optimum temperature for growth was 37 °C. Strain 4CA^T was resistant to erythromycin, bacitracin, novobiocin, polymyxin B, neomycin, gentamicin, streptomycin, carbenicillin, rifampicin and tetracycline, and was susceptible to nalidixic acid, chloramphenicol, ampicillin and penicillin. The isolate's 16S rRNA gene sequence indicated that it belonged to the γ -*Proteobacteria*, showing 90–94 % similarity to its closest relatives. Maximum-likelihood phylogenetic inferences placed strain 4CA^T within a novel lineage related to the marine bacterial genera *Neptunomonas* and *Marinobacterium*. The DNA G + C content of the isolate was 47.4 mol%. On the basis of genotypic and phenotypic characterization, it was concluded that strain 4CA^T should be placed in a separate taxon as a novel genus and species, with the proposed name *Nitrincola lacisaponensis* gen. nov., sp. nov. The type strain is 4CA^T (=ATCC BAA-920^T=DSM 16316^T).

Soda lakes are highly alkaline, naturally occurring environments that harbour a microbiota representing most major prokaryotic lineages (Zavarzin *et al.*, 1999). In particular, the majority of Gram-negative isolates and culture-independent bacterial clones retrieved from soda lakes belong to the γ -*Proteobacteria* (Jones *et al.*, 1994, 1998; Ma *et al.*, 2004),

including strains related closely to typical aquatic bacteria such as *Aeromonas* and *Pseudomonas* (Duckworth *et al.*, 1996), moderate halophiles from the *Halomonas/Deleya* group, and marine bacteria, e.g. *Marinobacter* (Rees *et al.*, 2004).

Of the phylogenetically diverse Gram-negative, marine and/or halophilic aerobes and facultative anaerobes, the genus *Oceanospirillum* is the most widely studied and has traditionally consisted of various types of bacteria that occur in marine ecosystems (Hylemon *et al.*, 1973). DNA–rRNA hybridization experiments (Pot *et al.*, 1989, 1992) and fatty acid analysis (Sakane & Yokota, 1994) have revealed much intragenetic diversity within this group. Recently, analyses based on 16S rRNA and *gyrB* genes helped to clarify the taxonomic affiliation of the oceanospirilla and related

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Abbreviations: CA, coumaric acid; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Nitrincola lacisaponensis* 4CA^T is AY567473.

bacteria (Satomi *et al.*, 2002), leading to the redefinition of this genus to include four species. Other, less-studied genera affiliated with *Oceanospirillum* include *Marinobacterium* and *Neptunomonas*. These marine bacteria display the capability to grow on a wide range of substrates, including some polycyclic aromatic hydrocarbons in the case of *Neptunomonas*, and are frequently isolated from creosote-contaminated marine environments (González *et al.*, 1997; Hedlund *et al.*, 1999; González & Whitman, 2002).

In this study, we report the isolation and characterization of a new bacterium, designated strain 4CA^T, obtained from Soap Lake, a meromictic lake with high alkalinity. Through the analysis of molecular and physiological properties, isolate 4CA^T was found to bear resemblance to the *Marinobacterium*–*Neptunomonas*–*Oceanospirillum* cluster, although it was distinct enough to be separated into a novel genus.

Isolate 4CA^T was obtained from decayed, red-coloured wood collected on the shore of Soap Lake, a meromictic, alkaline, saline lake located in Grant County, WA, USA. Soap Lake is a closed, evaporative system with neither a significant surface inlet nor outlet. This results in the concentration of salts, mainly sodium carbonate and sodium sulfate (Anderson, 1958). The current pH of the lake is about 9.8 and its NaCl concentration can exceed 10% (w/v) (Walker, 1974). Two sections of wood (approx. 1 cm²) were removed aseptically from the sample and added to Soap Lake basal medium (SLBM), an enrichment medium for putative moderately halophilic, alkaliphilic bacteria. SLBM contained the following (l⁻¹): CaSO₄, 4.0 mg; FeSO₄, 1.0 mg; NaCl, 17.5 g; SiO₂, 5.0 mg; MgCl₂, 4.0 mg; MnSO₄, 4.0 mg; NH₄NO₃, 50.0 mg; Na₂SO₄, 13.5 g; KH₂PO₄, 3.0 g; K₂HPO₄, 3.0 g; Na₂CO₃, 1.0 g; and 1 ml trace element stock solution [g l⁻¹: sodium nitriloacetate, 1.5; MgSO₄·7H₂O, 3.0; MnSO₄·H₂O, 0.5; NaCl, 1.0; FeSO₄·7H₂O, 0.1; CaCl₂·2H₂O, 0.1; CoCl₂·6H₂O, 0.1; ZnCl₂, 0.13; CuSO₄·5H₂O, 0.01; AlK(SO₄)₂·12H₂O, 0.01; H₃BO₃, 0.01; Na₂MoO₄·2H₂O, 0.025; NiCl₂·6H₂O, 0.024; Na₂WO₄·2H₂O, 0.025]. To enrich for bacteria capable of utilizing lignin-hydrolysis compounds, coumaric acid (CA) was added to a final concentration of 0.05% (w/v). The final pH of the medium was adjusted to 9.8 with 1 M NaOH. Cultures were placed in a shaking incubator (200 r.p.m.) at 37 °C. To obtain pure cultures, the enriched cells were streaked onto solid SLBM containing 0.05% (w/v) CA (pH 9.8). The strain selected for further study was maintained on solid SLBM or nutrient agar (Difco) slants.

The carbon-source utilization pattern was ascertained with a salt basal medium (SBM) described previously (Mormile *et al.*, 1999), except that the NaCl, yeast extract and NaNO₃ concentrations were lowered to 17.5, 0.1 and 0 g l⁻¹, respectively, and the pH was adjusted to 9.0. Each carbon source tested was added separately from sterile stocks to give a 1% final concentration, except for vanillic acid and CA, which were added at a concentration of 0.05%. A growth test was considered positive when the OD₆₀₀ reached or

exceeded a value of 0.3 after 72 h at 37 °C. SBM containing acetate was used to determine growth responses to pH by adjusting, for each test, the pH at values ranging from 6.0 to 13.0 by using a finely adjusted KH₂PO₄/K₂HPO₄ or Na₂CO₃/NaHCO₃ buffer system. The growth response to NaCl was measured by varying the NaCl concentration from 0 to 3.5 M. To determine whether the isolate required Na⁺ or Cl⁻, medium was prepared separately with KCl, Na₂SO₄, NaNO₃ or Na₂CO₃ to substitute for these ions. Tolerance to salt and pH values was determined by measuring OD₆₀₀ after 48 h incubation at 37 °C. Tolerance to temperature was determined after growth in SBM with acetate for 48 h. Potential electron acceptors other than oxygen were tested on SBM/acetate containing 0.001 g resazurin l⁻¹ and included NaNO₃, NaNO₂, Na₂SO₄, Na₂SO₃ and Na₂SeO₄, each supplied at a concentration of 10 mM. Anaerobic conditions were achieved as described by Cutter *et al.* (1998).

Gram reaction was determined with a Gram-stain kit (Difco). Catalase and oxidase activities, as well as endospore production, were determined as described by Smibert & Krieg (1994). Analyses for hydrolysis of starch and DNA were performed with starch agar (Difco) and DNase (Difco) media, respectively. The ability to reduce nitrate under aerobic conditions was assessed on nitrate broth (Difco) by following the manufacturer's recommendations. King's media A and B were used to determine the production of pigments (King *et al.*, 1954). Additional enzymic activities were assayed by using API 20E strips (bioMérieux). Antibiotic susceptibilities were determined by using antibiotic discs (Becton Dickinson) on SBM at pH 9.0.

For transmission electron microscopy (TEM), a bacterial cell pellet obtained from an overnight culture was fixed with 2% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer. After the samples were washed several times, 2% liquid agar was added and, upon solidification, 1 mm³ portions were post-fixed in 1% OsO₄. En bloc staining was done in 1% aqueous uranyl acetate. Samples were dehydrated in an ethanol series and infiltrated with Epon-Araldite epoxy resin. The material was then placed into fresh resin in BEEM embedding capsules and polymerized at 60 °C. After polymerization, blocks were sectioned on a Leica UCT ultramicrotome and ultrathin sections (70 nm thick) were stained with 1% uranyl acetate and lead citrate. Samples were viewed and photographed in a JEOL 1200EX transmission electron microscope and imaged on Kodak 4489 TEM film. To visualize flagella, a 5 µl aliquot of strain 4CA^T culture was placed on a Ni sample disc and evaporated to dryness. The sample was placed in a 27% solution of phosphotungstic acid for 30 s and rinsed with ultrapure water before TEM.

To determine the DNA base composition, DNA was extracted and purified by the method of Marmur (1961) and its G + C content was determined from the mid-point value (T_m) of the thermal-denaturation profile (Marmur & Doty, 1962) by using the equation of Owen & Hill (1979).

The 16S rRNA gene of strain 4CA^T was PCR-amplified with the broad-range bacterial primers 27F (5'-AGAGTTTGA-TCCTGGCTCAG-3'), 806R (5'-GGAACCTACAGGGTATC-TAAT-3'), 13B (5'-AGGCCCGGGAACGTATTTCAC-3') and 1527R (5'-AAGGAGGTGATCCAGCC-3') (Relman *et al.*, 1992; Weisburg *et al.*, 1991). PCR and sequencing reactions were performed by following the protocol described by Shukla *et al.* (2001). The sequencing reactions were resolved in an ABI 3100 gene analyser (Applied Biosystems). The assembled and edited 16S rRNA gene sequence of strain 4CA^T was aligned with 16S rDNA sequences of closely related strains by conducting BLAST searches of GenBank and performing multiple alignments using CLUSTAL W (Thompson *et al.*, 1994) with additional manual alignment. The phylogenetic relationships of each taxon were inferred by maximum-likelihood, parsimony and evolutionary-distance methods of phylogenetic reconstruction, using PAUP* 4.0 b10 (Swofford, 2003). Maximum-likelihood trees were inferred by using the default starting parameters (neighbour-joining using a Jukes–Cantor model of evolution). Statistical support of the branch points was tested by performing 1000 maximum-likelihood bootstrap replications. Parsimony trees were inferred by conducting heuristic searches with the tree-bisection–reconnection branch-swapping algorithm, all positions were weighted equally and gaps were treated as missing. Pairwise distances were used to generate neighbour-joining/UPGMA trees using Hasegawa, Kishino and Yano (HKG85) distance.

When grown for 2 days at 37 °C on solid SBM containing acetate, colonies of strain 4CA^T were typically white, circular and convex, with entire margins and a diameter of 1–2 mm. Colonies of the culture grown on nutrient agar (pH 9.8 and 1.75 % NaCl) exhibited similar morphology, but diameters were 3–4 mm. Liquid nutrient broth also supported growth of strain 4CA^T, although the bacteria had a slight tendency to clump as cultures aged. Cells were motile, short rods that possessed a single polar flagellum and stained Gram-negative; they were typically 1.3–1.6 µm long and 0.5 µm wide. Electron microscopy analyses confirmed the Gram-negative structure (Fig. 1) and position and number of the flagella (Fig. 2). In most cases, strain 4CA^T appeared as single cells. No endospore production was observed.

Fig. 1. Transmission electron micrograph of a thin section of cells of *Nitrincola laxisaponensis* 4CA^T. Bar, 0.2 µm.

Fig. 2. Transmission electron micrograph of cells of *Nitrincola laxisaponensis* 4CA^T, showing a single, polar flagellum. Bar, 1 µm.

Strain 4CA^T grew well in both liquid nutrient broth and solid nutrient agar with pH values ranging from 8.0 to 10.0. It could grow at NaCl concentrations of 0.2–1.3 M, with optimum growth occurring at 0.8 M NaCl. It could also grow at Na₂SO₄ concentrations of 0.2–1.0 M, NaNO₃ concentrations of 0.2–1.0 M and Na₂CO₃ concentrations of 0.2–1.2 M. When NaCl and other sources of Na⁺ were removed from the medium and replaced with KCl, isolate 4CA^T did not grow, indicating that it has a requirement for Na⁺. It grew best at 30–37 °C and some growth was detected at 22 °C, but it failed to grow at 4 °C. When tested on finely buffered SBM, strain 4CA^T had the ability to grow between pH values of 7.5 and 11.0, with optimal growth observed at pH 9.0. The final pH values measured in the media initially poised at 9.0, 9.5, 10.0, 10.5 and 11.0 were 9.08, 9.57, 9.95, 10.44 and 10.92, respectively. Hence, isolate 4CA^T could be considered a strict alkaliphile (Horikoshi, 1998; Ivey *et al.*, 1998).

The new strain was oxidase- and catalase-positive and did not exhibit lysine decarboxylase, ornithine decarboxylase or arginine dihydrolase activities. Strain 4CA^T was negative for the production of H₂S, Voges–Proskauer reaction and indole production; urea, gelatin, DNA and starch were not hydrolysed. Cells did not produce visible or fluorescent pigments when incubated on solid King's media B or A, respectively, two characteristics widely used for preliminary determination of pseudomonad species (Stanier *et al.*, 1966; Palleroni, 1984). Strain 4CA^T grew with O₂ or NO₂⁻ as electron acceptors in the presence of acetate, but not with NO₃⁻, SO₄²⁻, SO₃²⁻ or SeO₄²⁻. Under aerobic conditions, it utilized DL-lactate, acetate, pyruvate, malate, fumarate, succinate and Tween 60. Additional characteristics are provided in the species description.

The G+C content of the DNA of strain 4CA^T was 47.4 mol%, as determined by the thermal-denaturation method. The sequence of an approximately 1400 bp portion of the 16S rRNA gene was obtained for isolate 4CA^T. No

Fig. 3. Phylogenetic tree based on nearly complete 16S rRNA gene sequences of strain 4CA^T and its relatives in the γ -Proteobacteria. *Escherichia coli* was used to root the tree. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets; values of 50% or greater are shown. Bar, 2 nucleotide substitutions per 100 nt.

sequences available in the public databases exhibited >94% similarity to the sequence of strain 4CA^T. Phylogenetic analysis revealed that isolate 4CA^T clustered within the γ -subclass of the Proteobacteria, encompassing the genera *Neptunomonas*, *Marinobacterium* and *Oceanospirillum* (Fig. 3). Some pseudomonads belonging to the *Pseudomonas fluorescens* intrageneric cluster, one of the two major clusters that comprise the genus *Pseudomonas sensu stricto* (Moore *et al.*, 1996), formed a distinct subclade detached from strain 4CA^T. Maximum-likelihood, parsimony and distance methods resulted in highly similar tree topologies. Strain 4CA^T had a mean of 90–91.8% sequence similarity to all taxa that fell into this cluster, and displayed 94, 92.5 and 93% similarity to the marine bacteria *Marinobacterium* sp. strain DMS-S1 (Fuse *et al.*, 2000), *Neptunomonas naphthovorans* (Hedlund *et al.*, 1999) and *Oceanospirillum beijerinckii* (Hylemon *et al.*, 1973), respectively.

The phenotypic characteristics that differentiate strain 4CA^T from closely related taxa are presented in Table 1. The strain could be distinguished from members of the genus *Oceanospirillum* by its shape and its ability to grow in the absence of NaCl. Some phenotypic properties delineating strain 4CA^T were also in marked disagreement with those that characterize members of the genus *Marinobacterium*: the type species, *Marinobacterium georgiense*, can utilize sugars and amino acids, requires a salt-based medium for growth and grows best at pH 7.0 (González *et al.*, 1997; González & Whitman, 2002). A similar physiological profile is observed for members of the genus *Neptunomonas* (Hedlund *et al.*, 1999). None of the related genera can use NO₂⁻ as an electron acceptor, nor do they exhibit an alkaliphilic phenotype.

The results presented here provide phenotypic and molecular (Fig. 3) evidence that warrants the affiliation of strain 4CA^T to a separate, novel lineage, excluding it from any of the previously described genera of the γ -Proteobacteria to which it is phylogenetically related. Thus, we conclude that isolate 4CA^T represents a novel genus and species, for which

the name *Nitrincola laciasaponensis* gen. nov., sp. nov. is proposed.

Description of *Nitrincola* gen. nov.

Nitrincola (Nit.rin'co.la. L. neut. n. *nitrum* soda; L. masc. n. *incola* inhabitant, dweller; N.L. masc. n. *Nitrincola* an inhabitant of a soda environment).

Table 1. Phenotypic characteristics that differentiate strain 4CA^T from phylogenetically related taxa

Taxa: 1, *Nitrincola laciasaponensis* 4CA^T (this study); 2, *Oceanospirillum*; 3, *Pseudomonas mosselli* CFML 90-83^T (Dabboussi *et al.*, 2002); 4, *Neptunomonas*; 5, *Marinobacterium*. ND, No data; +, positive reaction; –, negative reaction. Data were taken from González & Whitman (2002) unless otherwise noted.

Characteristic	1	2	3	4	5
Cell morphology	Rods	Helical	Rods	Rods	Rods
Optimum pH	9.0	ND	ND	ND	7.5
Optimum growth temperature (°C)	37	30–32	30	30	37
Urease	–	–	ND	–	+
Arginine dihydrolase	–	ND	+	–	ND
Gelatinase	–	+	+	–	–
Pigment production	–	ND	+	ND	ND
DNA G + C content (mol%)	47.4	47–49	63	46	55
Utilization of:					
Galactose	–	–	–	+	ND
Glucose	–	–	+	+	+
D-Mannose	–	–	+	–	+
D-Fructose	–	–	+	+	+
DL-Lactate	+	+	+	ND	ND
Malate	+	+	+	–	ND
Ethanol	–	+	ND	ND	+
Glycerol	–	–	+	+	–
Propanol	–	+	ND	ND	+

Alkaliphilic, halotolerant and heterotrophic. Cells are non-pigmented, asporogenous, motile, Gram-negative rods. NO_2^- and O_2 can be used as electron acceptors. Fermentable carbon sources do not support growth. Chemorganotrophic. Requires sodium for growth. Oxidase- and catalase-positive. Optimal pH for growth is 9.0. The genus is a member of the γ -subclass of the *Proteobacteria*. Isolated from a saline, alkaline lake. The DNA G+C content is 47.4 mol% (T_m). The type species is *Nitrincola laxisaponensis*.

Description of *Nitrincola laxisaponensis* sp. nov.

Nitrincola laxisaponensis (la.ci.sa.po.nen'sis. L. n. *lacus* lake; L. n. *sapo* -onis soap; N.L. masc. adj. *laxisaponensis* pertaining to Soap Lake).

Cells are short, motile rods, occurring singly or in pairs (1.3–1.5 μm in length and 0.5 μm in width). When grown on alkaline nutrient agar, colonies are 3–4 mm in diameter, whitish-beige-coloured, entire, smooth and convex. Obligate alkaliphile. Oxidase and catalase are produced. Lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase are not produced; nor are H_2S , acetoin or indole. Urea, gelatin, DNA and starch are not hydrolysed. No visible or fluorescent pigments are formed during incubation on solid King's media B or A, respectively. Strain 4CA^T can grow with O_2 or NO_2^- as electron acceptors in the presence of acetate, but not with NO_3^- , SO_4^{2-} , SO_3^{2-} or Se_4^{2-} . Carbon-source utilization is restricted to a limited range of organic acids, including DL-lactate, acetate, pyruvate, malate, fumarate and succinate. Glycine, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamic acid, L-glutamine, L-isoleucine, L-leucine, L-ornithine, L-phenylalanine, sarcosine, L-threonine, L-serine, L-tryptophan, L-methionine, mannitol, D-mannose, D-arabinose, sucrose, D-xylose, glucose, D-galactose, cellobiose, D-fructose, D-ribose, ethanol, adonitol, glycerol, dulcitol, sorbitol, propanol, lactose, glucuronate, saccharate, hippurate, vanillate, coumarate and benzoate are not utilized. Cells are resistant to erythromycin, bacitracin, novobiocin, polymyxin B, neomycin, gentamicin, streptomycin, carbenicillin, rifampicin and tetracycline, and are susceptible to nalidixic acid, chloramphenicol, ampicillin and penicillin. In minimal medium (SBM) containing acetate, the conditions for growth are pH 7.5–11.0 and up to 1.3 M NaCl. Optimum growth occurs at pH 9.0 and at 0.8 M NaCl. The DNA G+C content is 47.4 mol% (T_m).

Isolated from decayed wood collected at Soap Lake, a saline, alkaline environment. The type strain is 4CA^T (=ATCC BAA-920^T=DSM 16316^T).

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