



## *Nitrincola nitratreducens* sp. nov. isolated from a haloalkaline crater lake<sup>☆</sup>

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### ABSTRACT

The novel, cream coloured, Gram-negative-staining, rod-shaped, motile bacteria, designated strains AK23<sup>T</sup> and AK28, were isolated from sediment samples collected from Lonar Lake, Buldhana district, India. The predominant fatty acids were C<sub>18:1</sub>ω7c, C<sub>16:0</sub>, C<sub>10:0</sub> 3OH and C<sub>16:1</sub>ω7c and/or iso-C<sub>15:0</sub> 2OH (summed feature 3). Polar lipid content of strains AK23<sup>T</sup> and AK28 were found to be phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), one unidentified phospholipid (PL) and two unidentified lipids (L1 and L2). The 16S rRNA gene sequence analysis indicated strains AK23<sup>T</sup> and AK28 as the members of the genus *Nitrincola* and closely related to the type strain *Nitrincola lacisaponensis* with pair-wise sequence similarity of 97.67% and 97.62% respectively. DNA–DNA hybridization between strain AK23<sup>T</sup> and AK28 showed a relatedness of 91%. Genome of strains AK23<sup>T</sup> and *N. lacisaponensis* DSM 16316<sup>T</sup> were sequenced. A comparative genomics approach was used to study strains AK23<sup>T</sup>, *N. lacisaponensis* DSM 16316<sup>T</sup> and five other phylogenetic neighbours. The genome size of *N. lacisaponensis* DSM 16316<sup>T</sup> was found to be 614,784 bp smaller than that of the strain AK23<sup>T</sup>. This variation could be due to multiple reasons, gene uptake, evolution, mutation, genome reduction phenomenon and draft nature of sequencing. Based on data from the current polyphasic study, strains AK23<sup>T</sup> and AK28 are proposed as novel species of the genus *Nitrincola*, for which the name *Nitrincola nitratreducens* sp. nov. is proposed. The type strain of *N. nitratreducens* is AK23<sup>T</sup> (=JCM 18788<sup>T</sup> = MTCC 11628<sup>T</sup>).

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The genus *Nitrincola* was established to accommodate a single species, *Nitrincola lacisaponensis* [8]. *N. lacisaponensis* was isolated from alkaline soda lake, the cells were Gram-negative, motile, asporogenous and rod shaped. The DNA G+C content of strain *N. lacisaponensis* is 47.4% [8]. The family *Oceanospirillaceae* was created in the year 2005, it consists of 18 genera, *Amphritea*, *Balneatrix*, *Bermanella*, *Corallomonas*, *Marinomonas*, *Marinospirillum*, *Neptuniibacter*, *Neptunomonas*, *Nitrincola*, *Oceaniserpentilla*, *Oceanobacter*, *Oceanospirillum*, *Oleibacter*, *Oleispira*, *Pseudospirillum*, *Reinekea*, *Sinobacterium* and *Thalassolituus* [10]. Lonar Lake, a haloalkaline lake was created by a meteorite impact, reported to

contain heavy metals and a harbouring wide bacterial diversity [5]. Many novel genera and species have been isolated from this lake [2–4,20,25]. In the present study, we focused on the characterization and classification of the strains AK23<sup>T</sup> and AK28, which were isolated from Lonar Lake sediment, by using a polyphasic approach [30]. From the results of phylogenetic and phenotypic analyses, the strains are proposed as the representatives of a novel species of the genus *Nitrincola*.

Strains AK23<sup>T</sup> and AK28 were isolated from the sediment samples collected from Lonar Lake, a haloalkaline lake situated in the Buldana district of Maharashtra, India on 19th of March 2012 (GPS position 19°58'17.18" N 76°30'22.57" E). The sediment sample from which AK23<sup>T</sup> and AK28 were isolated had a pH of 10, temperature of 32 °C and salinity 2.5%. Approximately 100 mg of the sediment sample was suspended in 900 ml of sterile saline water (2% NaCl w/v) and subjected to shaking for 2 h. The supernatant was serially diluted and 100 μl was plated on Zobell marine agar (MA) [34] plates and incubated at 30 °C for 15 days. Based on the colony morphology two off-white colonies were selected and characterized in the present study.

<sup>☆</sup> The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strains AK23<sup>T</sup> and AK18 are HE983365 and HG974241 respectively and whole genome sequence of strains AK23<sup>T</sup> and *Nitrincola lacisaponensis* DSM 16316<sup>T</sup> are AONB01000000 and JMSZ01000000 respectively.

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Strains AK23<sup>T</sup> and AK28 were characterized simultaneously with *N. laciaponensis* DSM 16316<sup>T</sup> that was procured from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. Colony morphology was examined following growth of the strains on MA at 30 °C for 24 h. Cell morphology and motility were observed by using phase contrast microscopy (Nikon). Motility was also assessed on Motility-Indole-Lysine HiVeg<sup>TM</sup> medium (HIMEDIA) with 0.2% agar (by inoculating the active culture suspension using sterile inoculation needle and checking for spreading of the growth in the medium). Growth at temperatures of 4, 10, 15, 20, 30, 37, 42 and 45 °C was ascertained using marine broth medium and salt tolerance [0,0.5, 1, 2, 3, 4, 5, 6 and 8% (w/v) NaCl] was ascertained using nutrient broth medium containing peptone (0.5%) and beef extract (0.3%). Growth of strains at pH 5, 6, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11 and 12 was assessed on marine broth buffered with citric acid/NaOH (for pH 5 and 6), NaHPO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> (for pH 7 and 8), glycine/NaOH (for pH 9 and 10) or Tris-HCl/NaOH (for pH 11 and 12). Biochemical characteristics such as activity of oxidase, catalase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, nitrate reduction, hydrolysis of aesculin, ONPG, H<sub>2</sub>S production and the sensitivity to 16 different antibiotics using the disc diffusion method with commercially available discs (HIMEDIA) were determined by previously described methods [15,24]. Amylase, lipase, caseinase, gelatinase, DNase and urease activities were determined using methods described earlier [26] with an exception that the cultures were grown on MA plates supplemented with other substrates and incubated at 30 °C for a week. Tweens 20, 40, 60, and 80 were separately used as substrates for evaluation of lipase activity. The zone of inhibition (sensitive or resistant) to the antibiotics was taken from the information given in the HIMEDIA product overleaf (manufacturer instructions). Biochemical and enzymatic characterization of the strains was also performed using Vitek 2 GN (bioMérieux) with incubation at 30 °C, according to the manufacturer's protocol, except that sterile 2.0% (w/v) NaCl was used to prepare the inoculum.

Chemotaxonomic characteristics like polar lipids, fatty acids, DNA G + C content were determined using methods described earlier [23,27]. The strains were grown on trypticase soy agar (TSA) medium with 2% (w/v) NaCl at 30 °C for 24–48 h and harvested at the same physiological age (at logarithmic phase of growth) for fatty acid analysis.

For 16S rRNA gene sequencing, DNA was prepared using a microbial DNA isolation kit (Mo Bio Laboratories Inc.) and sequenced as described previously [14]. The resultant sequences of the 16S rRNA gene (1458 and 1427 nt of strains AK23<sup>T</sup> and AK28, respectively) were subjected to BLAST (Basic Local Alignment Search Tool) sequence similarity searches [1] and the EzTaxon-e server [12] to identify the nearest taxa. To reveal the exact phylogenetic position of strains AK23<sup>T</sup> and AK28 in the domain *Bacteria*, the 16S rRNA gene sequences were aligned against an ARB dataset using the ARB programme package [17]. All 16S rRNA gene sequences of closely related members of the genera *Amphritea*, *Marinobacterium*, *Marinomonas* and *Nep-tunomonas* were downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov>) and aligned using the CLUSTAL.W programme within MEGA5 package [28]. Phylogenetic trees were constructed using tree-making algorithms, the neighbour joining, maximum-likelihood and maximum parsimony methods using the MEGA5 package. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [9]. The evolutionary distances were computed using the Kimura 2-parameter method [13] and are in the units of the number of base substitutions per site.

DNA–DNA hybridization was performed by the membrane filter method [29] as described previously [21,22]. REP, ERIC and BOX-PCR were performed according to the protocol described by Versalovic et al. [31]. RAPD-PCR was carried out using method described earlier [27].

The genome of strain AK23<sup>T</sup> was sequenced in house using Ion Torrent Personal Genome Machine (Life Technologies, USA). GS De Novo Assembler (version 2.7, F. Hoffmann-La Roche Ltd., Basel, Switzerland) was used to assemble the sequences into contigs. Annotation of the genome was done using Prokaryotic Genome Annotation System (PROKKA) pipeline (version 1.5.2, Torsten Seemann, Victorian Bioinformatics Consortium). The genome of the closest neighbour of species AK23<sup>T</sup> and the only other member of the genus *Nitricola*, *N. laciaponensis* DSM 16316<sup>T</sup>, was also sequenced for better comparative studies. The genome of *N. laciaponensis* DSM 16316<sup>T</sup> was sequenced in house using Illumina MiSeq (Illumina Inc.) platform. Assembly of the raw sequencing data was performed using CLCbio Workbench (v. 7.0, CLCbio, Denmark). Also, five neighbours of strain AK23<sup>T</sup>, for which whole genome data was available were selected for the comparative studies, namely, *Amphritea japonica* ATCC BAA-1530<sup>T</sup>, *Marinobacterium litorale* DSM 23545<sup>T</sup>, *Marinobacterium rhizophilum* DSM 18822<sup>T</sup>, *Marinobacterium stanieri* S30<sup>T</sup> and *Nep-tunomonas japonica* DSM 18939<sup>T</sup>. All these genomes, including *N. laciaponensis* DSM 16316<sup>T</sup> and strain AK23<sup>T</sup> were re-annotated using Rapid Annotation using Subsystem Technology (RAST) server (<http://rast.nmpdr.org/rast.cgi>). The seven genomes were subjected to COG (Clusters of Orthologous Groups) functional annotation using WebMGA server (<http://weizhong-lab.ucsd.edu/metagenomic-analysis/server/cog/>). Synteny between these genomes was searched using Sibelia (v. 3.0.6, <http://bioinf.spbau.ru/sibelia>). Synteny map was plotted using CIRCOS (v. 0.67, <http://www.circos.ca>). All seven genomes were subjected to *in-silico* DNA–DNA hybridization (DDH) using Genome-to-Genome Distance Calculator (GGDC) with genome of strain AK23<sup>T</sup> as reference [6].

Cells of strains AK23<sup>T</sup> and AK28 were Gram-staining-negative, motile rods, 0.6–1.1 µm wide and 1.8–2.5 µm long (Supplementary Fig. S1). Colonies were circular, cream coloured, dull, off white, dry and flat on marine agar. Both strains were positive for catalase and oxidase activities and were capable of reducing nitrate. Growth was observed at 15–42 °C (optimum 30 °C), 0–6% NaCl (optimum 2%) and pH 6–12 (optimum 8–9). Other physiological and biochemical characteristics, including Vitek 2 GN card results are presented in the species description and Table 1.

The cellular fatty acid composition of the strains AK23<sup>T</sup> and AK28 showed a spectrum of 6 fatty acids with a pronounced dominance of (>5%) C<sub>18:1ω7c</sub>, C<sub>16:0</sub>, C<sub>10:0</sub> 3OH and C<sub>16:1ω7c</sub> and/or iso-C<sub>15:0</sub> 2OH (summed feature 3) (Table 2). Unsaturated fatty acids constituted 74.8% of the total fatty acids. Compared with *N. laciaponensis* DSM 16316<sup>T</sup>, the composition of fatty acids differed considerably in strains AK23<sup>T</sup> and AK28 (Table 2). Major polar lipid composition (phosphatidylethanolamine [PE] and phosphatidylglycerol [PG]) was similar in all studied strains. The other common polar lipids in all strains studied include one unidentified phospholipid (PL1) and one unidentified lipid (L2) (Supplementary Fig. S2). The strains AK23<sup>T</sup> and AK28 differed from the strain *N. laciaponensis* DSM 16316<sup>T</sup> by the presence of phosphatidylserine [PS], one unidentified lipid (L1) and the absence of diphosphatidylglycerol [DPG] (Supplementary Fig. S2). The G + C content of DNA of strains AK23<sup>T</sup> and AK28 were 46.8 and 46.2 mol% respectively from the lab procedure.

The 16S rRNA gene sequence analysis placed strains AK23<sup>T</sup> and AK28 within the genus *Nitricola*. The results indicated that at the 16S rRNA gene sequence level, strains AK23<sup>T</sup> and AK28 were close to each other (with 99.9% pair-wise 16S rRNA gene

**Table 1**  
Comparative biochemical and physiological characteristics of strains AK23<sup>T</sup>, AK28 and *Nitriicola lacsaponensis* DSM 16316<sup>T</sup>.

Characteristic	<i>Nitriicola nitratireducens</i> AK23 <sup>T</sup>	<i>Nitriicola nitratireducens</i> AK28	<i>Nitriicola lacsaponensis</i> DSM 16316 <sup>T</sup>
Cell size (μm)	1.8–2.0	1.8–2.0	1.3–1.6
Colony characteristics	Circular, off white, dull, dry and flat	Circular, off white, dull, dry and flat	Circular, off white, shiny, moist and flat
Salinity growth range (%)	0–6	2–6	0–6
Optimum pH	8–9	8–9	9
G + C content of DNA (mol%)	46.8	46.2	47.4
<b>Biochemical characteristics</b>			
Nitrate reduction to nitrite	+	+	–
<b>Hydrolysis of</b>			
Gelatin	+	+	–
Tween 20	–	+	+
<b>Utilization of</b>			
Lactose	+	–	–
Xylose	–	–	+
Dextrose	+	+	–
Trehalose	–	–	+
Melibiose	+	–	+
Sucrose	+	+	–
L-Arabinose	–	–	+
Inulin	+	–	–
Sodium gluconate	+	–	–
Glycerol	+	+	–
Inositol	+	+	–
Mannitol	+	+	–
Arabitol	+	+	–
<b>Vitek GN information</b>			
α-Glucosidase	+	+	–
<b>Antibiotic susceptibility (μg per disc unless indicated)</b>			
Cefalexin (30)	R	R	S
Amoxycillin (30)	R	R	S
Tetracycline (30)	R	I	R
Chlortetracycline (30)	I	S	I
Penicillin-G (2 units)	R	R	S
Ceftazidime (30)	S	R	I

Data from the present study. All strains were rod shaped and motile, require 2% NaCl (w/v) for optimum growth, temperature growth range is 15–42 °C and optimum at 30 °C, pH growth range is 6–12; positive for oxidase, catalase, arginine dihydrolase, L-proline arylamidase, tyrosine arylamidase activities, methyl red reaction and L-malate assimilation, negative for Voges Proskauer's reaction, H<sub>2</sub>S and indole production, Ala-Phe-Pro-arylamidase, L-pyrrolydonyl-arylamidase, β-galactosidase, β-N-acetyl glucosaminidase, glutamyl arylamidase, γ-glutamyl-transferase, β-glucosidase, β-xylosidase, β-alanine arylamidase, lipase, urease, β-N-acetyl galactosaminidase, α-galactosidase, phosphatase, glycine arylamidase, ornithine decarboxylase, lysine decarboxylase, β-glucuronidase and glu-gly-arg-arylamidase activities, L-lactate and succinate alkalization, L-histidine and L-lactate assimilation, coumarate, O/129 resistance, ELLMAN reaction, aesculin, casein and tween 40/60/80 hydrolysis and fermentation of glucose. All three strains showed utilization of sorbitol, maltose and fructose, while they did not utilize galactose, raffinose, mannose, salicin, dulcitol, adonitol, erythritol, α-methyl-D-glucoside, D-cellobiose, D-glucose, D-maltose, D-mannose, D-sorbitol, D-tagatose, palatinose, citrate, malonate, 5-keto-D-gluconate, rhamnose, melezitose, α-methyl-D-mannoside, ONPG, D-arabinose, malonate and sorbose. All strains were susceptible to antibiotics (μg per disc unless indicated) enoxacin (10), spectinomycin (100), cephadroxil (30), novobiocin (30), and erythromycin (10), moderately susceptible to ampicillin (25), while all strains were resistant to bacitracin (8), methicillin (5), neomycin (30) and vancomycin (30). + positive; – negative, R: resistant, S: susceptible, I: intermediate.

sequence similarity) and to the single phylogenetic neighbour, *N. lacsaponensis* the sole member of the genus, with a pair-wise sequence similarity of 97.67 and 97.62% respectively. The pair-wise sequence similarity with the other closely related members of the genera *Amphritea*, *Marinobacterium*, *Marinomonas* and *Nep-tunomonas* was between 93.7–90.3%. Phylogenetic tree (based on the 16S rRNA gene sequences) obtained using the neighbour joining (Fig. 1) method revealed clustering of the strains AK23<sup>T</sup> and

**Table 2**  
Fatty acid profile of strains AK23<sup>T</sup>, AK28 and *Nitriicola lacsaponensis* DSM 16316<sup>T</sup>.

Fatty acid composition	<i>Nitriicola nitratireducens</i> AK23 <sup>T</sup>	<i>Nitriicola nitratireducens</i> AK28	<i>Nitriicola lacsaponensis</i> DSM 16316 <sup>T</sup>
C <sub>10:0</sub>	2.9	2.4	17.2
C <sub>10:0</sub> 3OH	5.3	4.7	25.0
C <sub>12:0</sub>	3.4	2.8	9.5
C <sub>16:0</sub>	14.6	14.0	6.6
C <sub>18:1ω7c</sub>	41.6	41.6	28.1
Summed feature 3	32.2	34.4	10.1

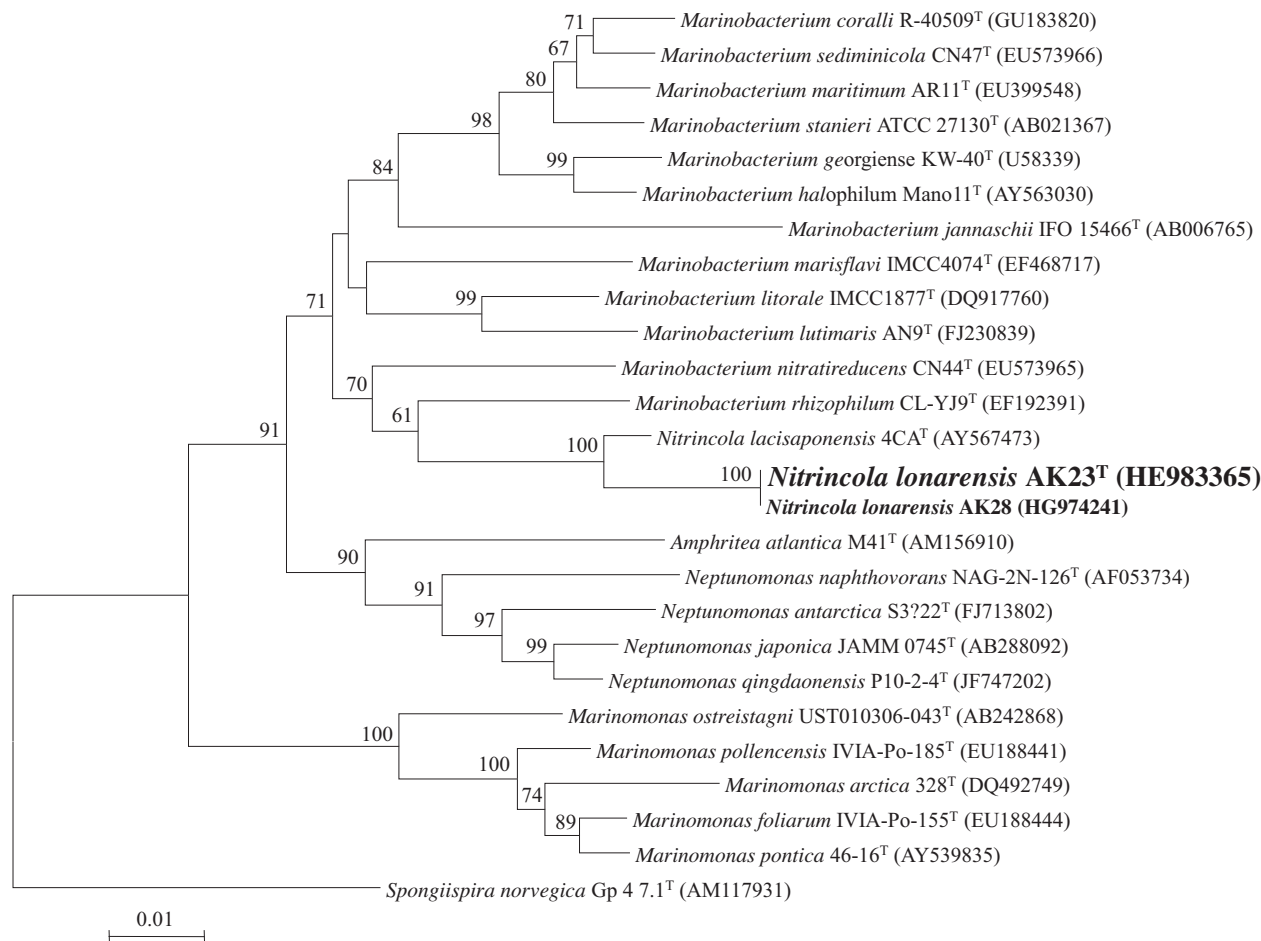
Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains C<sub>16:1ω7c</sub> and/or iso-C<sub>15:0</sub> 2OH.

Results are presented as a percentage of the total fatty acids. Fatty acids amounting to 5% or more of the total fatty acids are in bold. Data from the present study. The strains AK23<sup>T</sup>, AK28 and *N. lacsaponensis* DSM 16316<sup>T</sup> were grown on TSA plates with 2% (w/v) NaCl at 30 °C for 24–48 h and harvested at the same physiological age.

AK28 together and with *N. lacsaponensis* and all together clustered with members of the genus *Marinobacterium* (Fig. 1). DNA–DNA hybridization between strains AK23<sup>T</sup> and AK28 showed a reassociation of 91%. This value was above 70%, confirming that the novel strains are members of the same species [32]. DNA–DNA hybridization between strains AK23<sup>T</sup>, AK28 and *N. lacsaponensis* DSM 16316<sup>T</sup> showed a reassociation of 22.5 and 21.9% respectively. This value was below 70%, confirming that the novel strains are not members of the *N. lacsaponensis* DSM 16316<sup>T</sup> [32]. A number of differences were observed between the two strains AK23<sup>T</sup> and AK28 and also with type strain in all three Rep-PCR genomic fingerprint patterns (REP-, ERIC-, BOX-PCR) and RAPD-PCR genomic fingerprint patterns (Supplementary Fig. S3).

Whole genome sequencing of the strain AK23<sup>T</sup> yielded a total of 431,272,479 bases which is 107× coverage of the genome. Assembly of the raw sequencing data produced a genome of 4,027,646 bp in 68 contigs. All contigs were greater than 508 bp with the largest contig being 59,230 bp long. Similarly, whole genome sequencing of *N. lacsaponensis* DSM 16316<sup>T</sup> yielded a total of 544,056,464 bases which is 159× coverage of the genome. The assembly of the raw sequencing data generated a genome of 3,412,862 bp length in 44 contigs. All contigs were above the length of 503 bp with the largest contig of 816,335 bp. Annotation of strain AK23<sup>T</sup> consists of 4096 predicted protein encoding genes and 54 RNA features. The annotation of *N. lacsaponensis* DSM 16316<sup>T</sup> consists of 3093 predicted protein encoding genes and 53 RNA features. The genome size of strain AK23<sup>T</sup> is larger than that of *N. lacsaponensis* DSM 16316<sup>T</sup> by 614,784 bp and similarly it has 1004 annotation features more than the strain AK23<sup>T</sup>. The genomes of strain AK23<sup>T</sup> and *N. lacsaponensis* DSM 16316<sup>T</sup> is submitted to the NCBI GenBank database with accession numbers AONB01000000 and JMSZ01000000 respectively.

This variation in the genome sizes in the same genus can be attributed partially to the draft nature of sequencing which may miss some portion of the genome and partly to the fact that interspecies genome variations are possible. As there were no other discovered species under this genus, the comparative analysis is not sufficient. We cannot say this with surety if the strain AK23<sup>T</sup> has a larger genome than the average of *Nitriicola* genus or the bacterium *N. lacsaponensis* DSM 16316<sup>T</sup>. When any other member of the genus *Nitriicola* will be discovered, it can be sequenced and compared with the two already sequenced and described in this paper. It will lead us to trace the evolutionary map of the bacterium and to know if it is genome reduction in *N. lacsaponensis* DSM 16316<sup>T</sup> or gene uptake by the strain AK23<sup>T</sup>. There is also a possibility that the genome size variation is a result of draft nature of sequencing of the bacterium. But, the raw sequencing reads were 159× coverage of the genome, thus, chances of missing any substantial amount of genome data in sequencing is quite less. The



**Fig. 1.** Neighbour joining tree showing phylogenetic relationships of strain AK23<sup>T</sup> with closely related type strains of the family *Oceanospirillaceae* based on 16S rRNA sequence dataset. Bootstrap support values more than 50% are shown at the nodes. *Spongiispira norvegica* Gp 47.1<sup>T</sup> was used as out group taxon. Scale bar represents 0.01 substitutions per nucleotide position.

**Table 3**  
COG annotations of strain AK23<sup>T</sup> and its 6 phylogenetic neighbours.

COG class	Description	1	2	3	4	5	6	7
A	RNA processing and modification	1	1	2	1	1	1	1
B	Chromatin structure and dynamics	1	2	2	2	1	2	1
C	Energy production and conversion	235	283	311	281	253	184	244
D	Cell cycle control, cell division, chromosome partitioning	36	47	41	37	39	38	58
E	Amino acid transport and metabolism	277	337	442	339	378	316	363
F	Nucleotide transport and metabolism	66	83	101	79	77	76	86
G	Carbohydrate transport and metabolism	109	138	341	141	125	108	165
H	Coenzyme transport and metabolism	143	150	198	160	165	127	171
I	Lipid transport and metabolism	123	133	168	175	136	91	111
J	Translation, ribosomal structure and biogenesis	184	180	186	183	190	178	199
K	Transcription	243	280	342	293	307	194	257
L	Replication, recombination and repair	129	190	151	152	159	127	163
M	Cell wall/membrane/envelope biogenesis	177	159	224	175	202	178	213
N	Cell motility	92	131	72	128	116	126	138
O	Posttranslational modification, protein turnover, chaperones	147	167	170	151	159	127	151
P	Inorganic ion transport and metabolism	146	200	204	203	171	204	233
Q	Secondary metabolites biosynthesis, transport and catabolism	78	80	133	118	95	54	84
R	General function prediction only	379	421	509	422	432	325	418
S	Function unknown	314	300	368	308	311	242	314
T	Signal transduction mechanisms	297	394	314	404	362	334	412
U	Intracellular trafficking, secretion and vesicular transport	91	100	99	99	101	89	95
V	Defense mechanisms	34	37	56	37	46	37	60
Z	Cytoskeleton	0	0	0	0	0	0	1
	Total Features in COGs	3302	3813	4434	3888	3826	3158	3945

Taxa: 1. *Amphritea japonica* ATCC BAA-1530<sup>T</sup>; 2. *Marinobacterium litorale* DSM 23545<sup>T</sup>; 3. *Marinobacterium rhizophilum* DSM 18822<sup>T</sup>; 4. *Marinobacterium stanieri* S30<sup>T</sup>; 5. *Neptunomonas japonica* DSM 18939<sup>T</sup>; 6. *Nitrincola lacisaponensis* DSM 16316<sup>T</sup>; 7. *Nitrincola nitratireducens* sp. nov. AK23<sup>T</sup>.

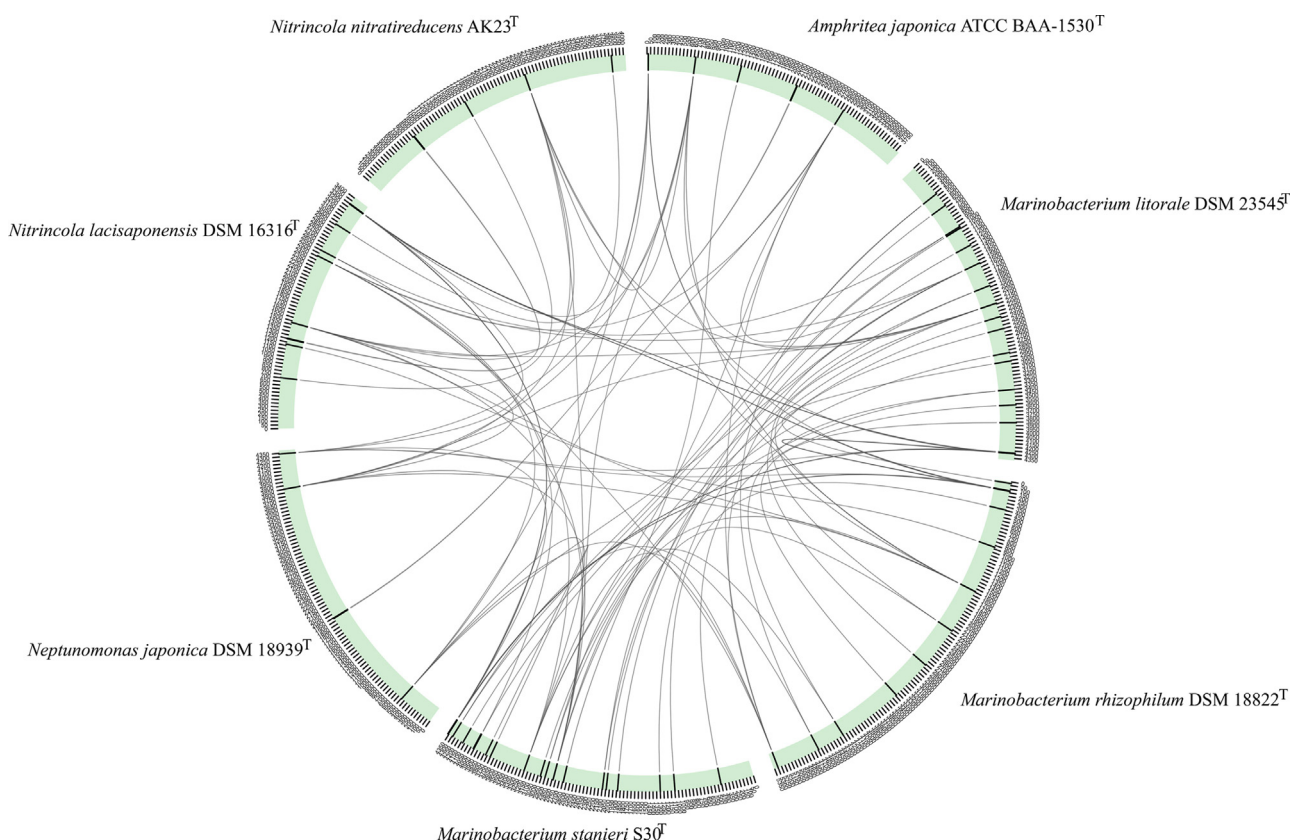


Fig. 2. CIRCOS image for the Synteny mapping performed by Sibelia. Length of mappings is given in Table 5.

variation in the size could be due to evolution, mutation and/or uptake of foreign DNA by the bacteria with larger genome. Generally, genome size tends to increase over evolutionary times due to gene duplications and increase in genetic elements. The opposite of this phenomenon, genome reduction, is also possible. Although, this phenomenon is more prevalent in endosymbionts [19] but also true for free living bacteria. The evolutionary process may sometimes lead to loss of genes which are generally 'accessory' genes, the genes not needed to survive in the present environment. This gene loss may be driven by drift or selection [16]. Also, among the studied bacteria, the largest genome size was of *A. japonica* ATCC BAA-1530<sup>T</sup> (5.36 Mb), followed by very similar genome sizes of *N. japonica* DSM 18939<sup>T</sup> (4.39 Mb), *M. stanieri* S30<sup>T</sup> (4.37 Mb) and *M. litorale* DSM 23545<sup>T</sup> (4.37 Mb). Smallest genome was of *N. lacisaponensis* DSM 16316<sup>T</sup> (3.4 Mb), followed by *A. japonica* ATCC BAA-1530<sup>T</sup> (3.79 Mb) and strain AK23<sup>T</sup> (4.02 Mb). On the analysis of annotation of the strain AK23<sup>T</sup> and *N. lacisaponensis* DSM 16316<sup>T</sup> reported the presence of genes for Colicin V and bacteriocin production, copper homeostasis, resistance against cobalt, zinc, cadmium,

mercury and arsenic, flagellar motility, heat shock, cold shock, detoxification and ABC transporters. Apart from that, the strains AK23<sup>T</sup>, *M. rhizophilum* DSM 18822<sup>T</sup> and *M. litorale* DSM 23545<sup>T</sup>, were found to consist of CRISPRs (clustered regularly interspaced short palindromic repeats). CRISPRs, in association with cas genes, make up the immune system that confers resistance to foreign genetic elements such as plasmids and phages [7]. Since 2012, CRISPRs/Cas system is being used for gene editing. This system can even be used for eukaryotes [33]. All except *M. rhizophilum* DSM 18822<sup>T</sup>, report the presence of genes for arsenic resistance. Cobalt–Zinc–Cadmium resistance genes were found in all seven bacteria under study, with strain AK23<sup>T</sup> having highest number of such genes. Siderophores, which help in iron acquisition, were found to be present in *N. lacisaponensis* DSM 16316<sup>T</sup>, *M. stanieri* S30<sup>T</sup> and *M. rhizophilum* DSM 18822<sup>T</sup>. Siderophores are small iron chelating compounds with high affinity. They are one of the strongest Fe<sup>3+</sup> binding agents [11,18].

The COG annotation divided all but strain AK23<sup>T</sup> into 22 different COG classes, strain AK23<sup>T</sup> was divided into a total of 23 COG

Table 4

*In silico* DNA–DNA hybridization results. Only the results of formula 2 are shown.

Reference genome	DDH	Model C.I.	Distance	Prob. DDH ≥70%
<i>Nitrincola lacisaponensis</i> DSM 16316 <sup>T</sup>	21.4	2.592834	0.2054	0
<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	19.1	2.543672	0.2297	0
<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	18.4	2.539616	0.2386	0
<i>Amphritea japonica</i> ATCC BAA-1530 <sup>T</sup>	18.5	2.541428	0.238	0
<i>Neptunomonas japonica</i> DSM 18939 <sup>T</sup>	18.2	2.541535	0.2415	0
<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	19.4	2.539518	0.227	0
Self ( <i>Nitrincola nitratireducens</i> AK23 <sup>T</sup> )	100	Inf	0	98.3

Query genome was the strain AK23<sup>T</sup>.  
Model C.I.: Model Confidence Interval.  
DDH: DNA–DNA Hybridization.  
Inf: infinite.

**Table 5**  
Synteny mapping performed by Sibelia software.

S. no.	Mapping between bacteria	Strand	Start	End	Length
1.	<i>Amphritea japonica</i> ATCC BAA-1530 <sup>T</sup>	+	669,346	672,792	3447
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	2,186,120	2,189,562	3443
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	–	1,593,227	1,589,750	3478
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	–	2,787,572	2,784,112	3461
	<i>Neptunomonas japonica</i> DSM 18939 <sup>T</sup>	+	3,850,588	3,854,041	3454
	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	+	1,475,049	1,478,532	3484
2.	<i>Nitricola nitratireducens</i> AK23 <sup>T</sup>	+	2,614,864	2,618,345	3482
	<i>Amphritea japonica</i> ATCC BAA-1530 <sup>T</sup>	+	2,840,665	2,843,089	2425
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	–	1,590,943	1,588,443	2501
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	+	5299914	5,302,419	2506
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	3,201,565	3,204,089	2525
	<i>Neptunomonas japonica</i> DSM 18939 <sup>T</sup>	–	494,889	492,425	2465
3.	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	+	2,458,230	2,460,695	2466
	<i>Amphritea japonica</i> ATCC BAA-1530 <sup>T</sup>	+	17,182	19,311	2130
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	4,280,038	4,282,193	2156
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	–	128,927	126,849	2079
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	–	4,363,380	4,361,201	2180
	<i>Neptunomonas japonica</i> DSM 18939 <sup>T</sup>	+	4,339,863	4,342,027	2165
4.	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	+	3,297,425	3,299,574	2150
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	4,276,969	4,279,298	2330
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	–	132,017	129,709	2309
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	–	4,366,305	4,364,105	2201
	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	+	3,294,430	3,296,670	2241
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	–	4,285,699	4,283,017	2683
5.	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	+	123,238	125,992	2755
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	4,357,712	4,360,395	2684
	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	–	3,303,083	3,300,416	2668
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	–	468,922	466,768	2155
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	–	4,689,772	4,691,808	2037
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	4,288,673	4,290,825	2153
7.	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	1,927,575	1,930,188	2614
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	+	2,218,439	2,220,952	2514
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	2,094,741	2,097,209	2469
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	–	3,401,531	3,399,314	2218
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	+	4,299,246	4,301,449	2204
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	–	2,901,315	2,899,095	2221
9.	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	1,019,028	1,021,282	2255
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	3,952,580	3,954,735	2156
	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	+	2,548,410	2,550,620	2211
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	–	3,859,655	3,856,817	2839
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	+	3,384,630	3,387,503	2874
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	–	2,890,880	2,888,449	2432
11.	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	–	2,965,576	2,968,000	2425
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	3,004,719	3,007,142	2424
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	–	23,659	21,239	2421
	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	+	1,243,640	1,252,708	9069
	<i>Nitricola nitratireducens</i> AK23 <sup>T</sup>	+	864,357	873,457	9101
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	+	392,651	395,325	2675
14.	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	1,096,984	1,099,599	2616
	<i>Amphritea japonica</i> ATCC BAA-1530 <sup>T</sup>	+	2,115,084	2,123,816	8733
	<i>Neptunomonas japonica</i> DSM 18939 <sup>T</sup>	+	1,938,626	1,947,347	8722
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	–	1,314,939	1,312,335	2605
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	1,886,126	1,888,730	2605
	<i>Amphritea japonica</i> ATCC BAA-1530 <sup>T</sup>	+	1,307,487	1,310,501	3015
17.	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	2,640,154	2,643,171	3018
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	667,270	669,404	2135
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	4,126,618	4,128,746	2129
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	2,548,767	2,550,940	2174
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	–	2,811,823	2,809,650	2174
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	2,379,057	2,381,260	2204
20.	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	–	1,300,848	1,298,666	2183
	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	–	703,674	701,311	2364
	<i>Nitricola nitratireducens</i> AK23 <sup>T</sup>	+	1,711,145	1,713,509	2365
	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	+	2,961,185	2,963,603	2419
	<i>Nitricola nitratireducens</i> AK23 <sup>T</sup>	+	3,832,347	3,834,760	2414
	<i>Marinobacterium rhizophilum</i> DSM 18822 <sup>T</sup>	+	924,216	926,894	2679
23.	<i>Nitricola lacisaponensis</i> DSM 16316 <sup>T</sup>	+	1,168,296	1,170,974	2679
	<i>Marinobacterium litorale</i> DSM 23545 <sup>T</sup>	+	1,040,930	1,043,063	2134
	<i>Marinobacterium stanieri</i> S30 <sup>T</sup>	+	2,042,647	2,044,762	2116

classes with 22 classes same as for the other genomes and one extra Cytoskeleton class with a single assigned feature (Table 3). Apart from that, the COG assignments were similar for all the genomes which is a notable point. For the synteny comparison, the

genomes were concocted into one single contig using EMBOS tools (v. 6.5.7, <http://emboss.sourceforge.net/>). Sibelia software was run with parameters “-s far-m 1000 to 5000”. The best synteny mapping was found at block size of 2000. CIRCOS was used to create

a circular map of the comparative data. Scalable Vector Graphics (SVG) image was created by CIRCOS which was edited to remove self-mappings. Only the inter-genomic mappings are shown in the Fig. 2. This suggests a close ancestral origin for these two bacteria. GGDC uses three formulae to calculate DDH distance out of which, formula 2 is recommended by the authors. This formula calculates the sum of all identities found in HSPs (High Scoring Segment Pairs) divided by the overall HSP length. Formula 2 is recommended as it is independent of genome length and hence better for incomplete draft genomes [6]. GGDC results clearly delineate the strain AK23<sup>T</sup> as the strain shares no significant DDH (minimum similarity of 70%) with any of its neighbours (Table 4).

The characteristics that differentiated strains AK23<sup>T</sup> and AK28 from the type strain *N. laciaponensis* DSM 16316<sup>T</sup> are given in Table 1. Although the strains AK23<sup>T</sup> and AK28 are in line with the genus *Nitricola*, they differed with the *N. laciaponensis* DSM 16316<sup>T</sup> in many characteristics (Table 1). Based on the blast and phylogenetic analysis, a detailed comparison was made between the characteristics of strains AK23<sup>T</sup> and AK28 with the type strain *N. laciaponensis* DSM 16316<sup>T</sup> of the family *Oceanospirillaceae* (Tables 1 and 2) and a number of differences were observed. For instance, strains AK23<sup>T</sup> and AK28 differed from *N. laciaponensis* DSM 16316<sup>T</sup> with respect to cell size, colony characteristics, salinity growth range, optimum pH, nitrate reduction, hydrolysis of gelatin and Tween 20, utilization of a number of carbon substrates,  $\alpha$ -glucosidase activity, antibiotic susceptibility, DNA G + C content, fatty acid and polar lipid composition (Tables 1 and 2, Supplementary Fig. S2). Thus, the cumulative differences that strain AK23<sup>T</sup> exhibits with the above closely related type strain unambiguously supports the creation of a new species of the genus *Nitricola* for which the name *Nitricola nitratireducens* sp. nov. is proposed.

### Description of *N. nitratireducens* sp. nov

*N. nitratireducens* (ni.tra.ti.re.du'cens. N.L. n. nitratum nitrate; L. v. reduco to draw backwards, bring back to a state or condition; N.L. part. adj. *nitratireducens* nitrate-reducing).

Cells are Gram-negative-staining, motile, aerobic, rod shaped, 0.6–1.1  $\mu\text{m}$  wide and 1.8–2.5  $\mu\text{m}$  long. The colonies on marine agar plate are circular, dull, dry, flat and off white coloured. Growth is observed at 15–42 °C (optimum 30 °C) and at pH 6–12 (optimum 8–9). Strains tolerate up to 6% NaCl (optimum 2%). Strains are capable of growth in medium that has not been supplemented with additional NaCl. Hydrolyze gelatin and aesculin. Oxidase, catalase, arginine dihydrolase,  $\alpha$ -glucosidase, L-proline arylamidase and tyrosine arylamidase activities are present but negative for Ala-Phe-Pro-arylamidase, L-pyrrolydonyl-arylamidase,  $\beta$ -galactosidase,  $\beta$ -N-acetyl glucosaminidase, glutamyl arylamidase,  $\gamma$ -glutamyl-transferase,  $\beta$ -glucosidase,  $\beta$ -xylosidase,  $\beta$ -alanine arylamidase, lipase, urease,  $\beta$ -N-acetylgalactosaminidase,  $\alpha$ -galactosidase, phosphatase, glycine arylamidase, ornithine decarboxylase, lysine decarboxylase,  $\beta$ -glucuronidase and glu-gly-arg-arylamidase activities. Positive for nitrate reduction and methyl red reaction, negative for Voges Proskauer's reaction, H<sub>2</sub>S and indole production, L-lactate and succinate alkalization, courmarate, 0/129 resistance, ELLMAN reaction, tween 40/60/80 hydrolysis. Utilize dextrose, sucrose, glycerol, inositol, L-malate, mannitol and arabinol, but not able to utilize xylose, trehalose, L-arabinose, dulcitol, rhamnose, cellobiose, melezitose,  $\alpha$ -methyl-D-mannoside, D-arabinose, citrate, malonate, sorbose, galactose, raffinose, mannose, salicin, adonitol, erythritol,  $\alpha$ -methyl-D-glucoside, D-cellobiose, D-glucose, D-maltose, D-mannose, D-sorbitol, D-tagatose, D-trehalose, 5-keto-D-gluconate, L-lactate, palatinose, L-histidine and ONPG. Only strain AK23<sup>T</sup> can utilize

lactose, melibiose, inulin, sodium gluconate and xylitol. Susceptible to ( $\mu\text{g}$  per disc unless indicated) ceftazidime (30), cephadroxil (30), enoxacin (10), erythromycin (10), novobiocin (30) and spectinomycin (100), moderately susceptible to ampicillin (25) and chlortetracycline (30) and resistant to amoxicillin (30), bacitracin (8), cefalexin (30), methicillin (5), neomycin (30), penicillin-G (2 units), tetracycline (30) and vancomycin (30). The major fatty acids are C<sub>18:1 $\omega$ 7c</sub>, C<sub>16:0</sub>, C<sub>10:0</sub> 3OH and C<sub>16:1 $\omega$ 7c</sub> and/or iso-C<sub>15:0</sub> 2OH (summed feature 3). The polar lipids include phosphatidylethanolamine, phosphatidylglycerol, phosphotidylserine, one unidentified phospholipid and two unidentified lipids. The G + C content of DNA of the strains AK23<sup>T</sup> and AK28 were 46.8 and 46.2 mol%.

The type strain AK23<sup>T</sup> (=JCM 18788<sup>T</sup> = MTCC 11628<sup>T</sup>), and reference strain AK28 were isolated from sediment sample collected from Lonar lake, Lonar, Maharashtra, India. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AK23<sup>T</sup> is HE983365 and whole genome sequence of strain AK23<sup>T</sup> is AONB01000000.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.syapm.2015.09.002>.

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