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Nesterenkonia lacusekhoensis sp. nov., isolated from hypersaline Ekho Lake, East Antarctica, and emended description of the genus *Nesterenkonia*

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An aerobic and heterotrophic isolate, designated IFAM EL-30^T, was obtained from hypersaline Ekho Lake (Vestfold Hills, East Antarctica). The isolate consisted of Gram-positive cocci or short rods which occasionally exhibited branching. The organism was moderately halotolerant, required thiamin.HCl and was stimulated by biotin and nicotinic acid. It grew well with glucose, acetate, pyruvate, succinate, malate or glutamate, and hydrolysed DNA but not gelatin, starch or Tween 80. Nitrate was aerobically reduced to nitrite. Chemical analysis revealed diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine and an unidentified glycolipid as the major polar lipids. The cellular fatty acids were predominantly of the anteiso and iso methyl-branched types, and the major menaquinones were MK-7 and MK-8. The peptidoglycan type was A4α, L-Lys-L-Glu. The DNA base ratio was 66·1 mol% G+C. Comparisons of 16S rRNA gene sequences showed that the unidentified organism was phylogenetically closely related to *Nesterenkonia halobia*, although a sequence divergence value of >3% demonstrated that the organism represents a different species. On the basis of phenotypic and genotypic evidence, it is proposed that the unknown bacterium be designated as a new species of the genus *Nesterenkonia*, namely *Nesterenkonia lacusekhoensis* sp. nov., the type strain being IFAM EL-30^T (= DSM 12544^T = CIP 107030^T). An emended description of the genus *Nesterenkonia* is given.

Keywords: *Nesterenkonia lacusekhoensis* sp. nov., Antarctica, hypersaline lakes, phylogeny, 16S rRNA

INTRODUCTION

The hypersaline, meromictic and heliothermal Ekho Lake in the ice-free Vestfold Hills (East Antarctica) has its greatest depth at 42 m and an oxylimnion of 24 m (Labrenz *et al*., 1998). In the summer of 1990, the salinity was found to increase stepwise from approx. 2‰ (surface) to > 180‰ (bottom), and Secchi disk visibility was 10 m (Lawson *et al*., 2000). These physical and chemical changes with depth offered micro-organisms a great variety of microhabitats (Labrenz & Hirsch, 2001). An attempt to characterize the aerobic microbial diversity of Ekho Lake in 1990 resulted in the isolation of 250 strains (P. Hirsch, J. Siebert & H. R. Burton, unpublished data). Upon closer investigation of 52 isolates by partial 16S rDNA sequencing and with biochemical and chemotaxonomic methods, only one strain corresponded to a known species, *Hyphomonas jannaschiana*; all other isolates represented new taxa. Several of these have recently been assigned to new genera [*Antarctobacter heliothermus* (Labrenz *et al*., 1998), *Roseovarius tolerans* (Labrenz *et al*., 1999) or *Staleya guttiformis* (Labrenz *et al*., 2000)] or have been described as new species [*Sulfitobacter brevis* (Labrenz *et al*., 2000), *N. aquaticus* and *F. lacustris* (Lawson *et al*., 2000)]. In the

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present study, we report the characteristics of another new Gram-positive species which was obtained from a 23 m water sample of Ekho Lake.

METHODS

Culturing techniques and tests. Water sampling, enrichments, growth and microscopic studies were as described previously (Labrenz *et al*., 1998). All biochemical tests were performed in triplicate. Oxidase, peroxidase and catalase activities, as well as sulfide or acetoin production, were determined according to the procedures described by Labrenz & Hirsch (2001). Temperature, artificial sea water, NaCl and pH-tolerance ranges for growth were studied as previously described by Labrenz *et al*. (1998). Tests for oxygen requirements, nitrate reduction, gelatin liquefaction and the hydrolysis of DNA and starch were carried out according to Labrenz *et al*. (1998) and Labrenz & Hirsch (2001). The aerobic dissimilation of carbon compounds was investigated by employing concentrations of 0·2 % (w/v) or by using the Biolog and API 50CH systems (Labrenz *et al*., 2000). Vitamin requirements were identified using three serial transfers as described previously (Labrenz *et al*., 1998). Antibiotic sensitivity was tested on agar plates with bioDiscs (bioMérieux) and the results recorded after 4 days.

Lipid analyses. The analysis of fatty acid methyl esters was performed by GC after acid hydrolysis of ester- and amide-linked fatty acids (Labrenz *et al*., 1998). Respiratory lipoquinones and polar lipids were extracted and analysed as described by Tindall (1990a, b).

Cell-wall analyses and determination of DNA base composition. Purified cell-wall preparations were made, and cell-wall hydrolysates analysed, according to Schleifer & Kandler (1972) with the modifications described by Willems *et al*. (1997). The G+C content of DNAs were determined by using HPLC after digestion of DNAs with P1 nuclease and alkaline phosphatase (Mesbah *et al*., 1989).

16S rRNA gene sequence analysis. 16S rRNA gene fragments were generated by using a PCR, as described by Hutson *et al*. (1993). An almost complete fragment of the 16S rRNA gene was amplified from DNA by using a PCR with universal primers pA (positions 8–28, *Escherichia coli* numbering) and pH* (positions 1542–1522). The amplified product was purified with a PCR purification kit (Qiagen) and sequenced directly using primers to conserved regions of the rRNA. Sequencing occurred with an Applied Biosystems' PRISM *Taq* DyeDeoxy Terminator Cycle Sequencing kit and model 373A automatic DNA sequencer. The closest relatives of strain EL-30^T were determined by performing EMBL-database searches with the the FASTA program (Pearson & Lipman, 1988). The sequences were aligned with the newly developed sequences by using the PILEUP program (Devereux *et al*., 1984). The RNA alignment was corrected manually and the (approximately) 100 highly variable bases of the 5′ end omitted. Percentage sequence similarities were corrected for substitution rates according to Jukes & Cantor (1969). A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the NEIGHBOR program. For this purpose, the following additional taxa were included: *Nesterenkonia halobia* (X80747), *Kocuria kristinae* (X80749), *Rothia nasimurinum* (AJ131121), *Rothia dentocariosa* I (M59055), *Rothia mucilaginosa* (X87758), *Kocuria palustris* (Y16263), *Kocuria varians* (X87754), *Kocuria rhizophila* (Y16264), *Kocuria rosea* (X87756), *Arthrobacter atrocyaneus* (X80746), *Arthrobacter cumminsii* (X93354), and, as the outgroup, *Renibacterium salmoninarum* (X51601). The stability of the groupings was estimated by bootstrap analyses (500 replications) using the programs of the PHYLIP package (Felsenstein, 1989). In addition, a parsimony analysis was performed with the same dataset (Felsenstein, 1989).

RESULTS AND DISCUSSION

Isolation, morphology and growth

Strain IFAM EL-30^T was isolated in January 1990 from a 23 m water sample of Ekho Lake. The sample had a salinity of 140‰, a temperature of 15·4 °C and a pH of 6·9. The isolate consisted of Gram-positive, short rods which showed occasional branching (Fig. 1, top); older cultures consisted largely of cocci (Fig. 1, bottom). Cells were non-motile and did not contain poly-β-hydroxybutyrate storage granules. Agar colonies were bright yellow and grew optimally in medium PYGV (Staley, 1968) at 27–33·5 °C, at pH 8·6 and in the presence of 6–8 % NaCl (or 50–90‰ artificial sea water, respectively). Thiamin.HCl was required, and biotin as well as nicotinic acid stimulated growth. The pH-tolerance range for growth was 7·5–9·5. The cells were sensitive to chloramphenicol, penicillin G or streptomycin and resistant to tetracycline or polymyxin B.

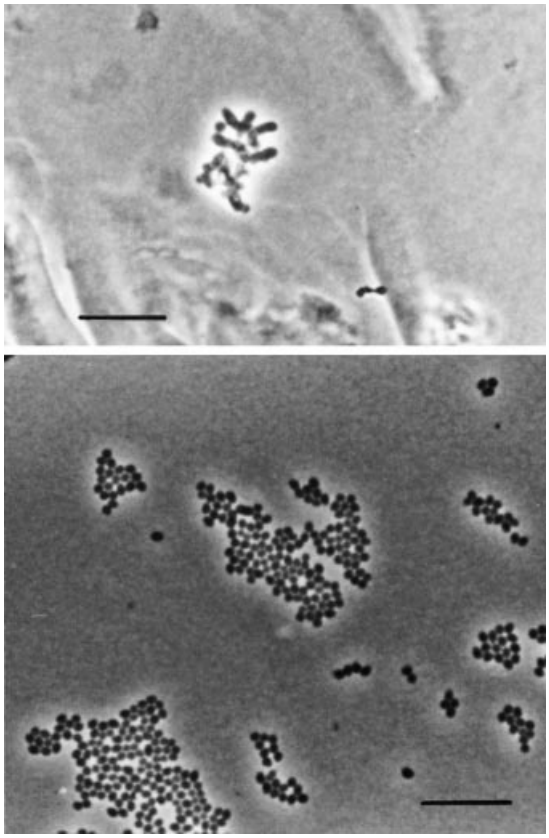

Fig. 1. Phase-contrast light micrographs showing the morphology of *Nesterenkonia lacusekhoensis* IFAM EL-30^T. (top) Young cell aggregate showing branching. (bottom) Irregular cocci or short rods of an older culture. Bars, 10 μm.

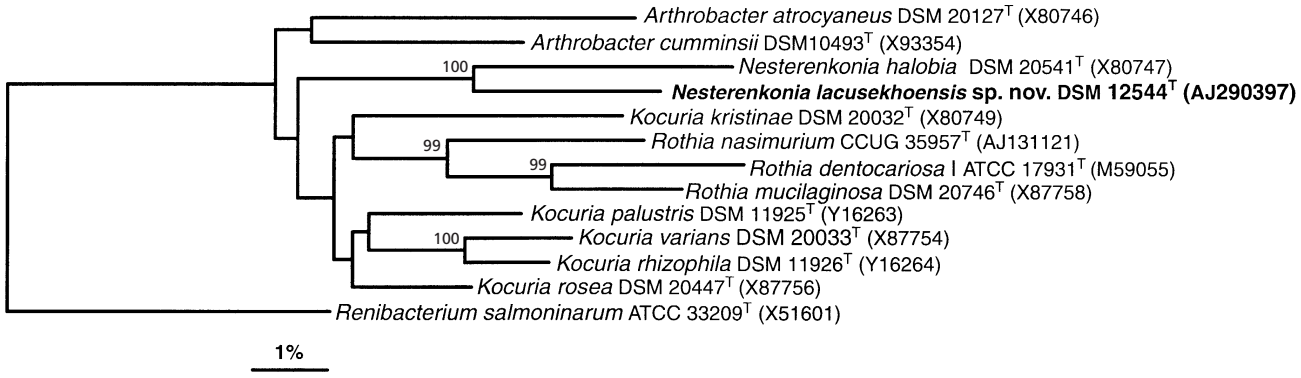

Fig. 2. Dendrogram showing phylogenetic relationships of isolate *Nesterenkonia lacusekhoensis* IFAM EL-30^T with respect to *Nesterenkonia halobia* and other closely related bacteria. The tree was constructed using the neighbour-joining method, and bootstrap values are expressed as percentages of 500 replications (only those values above 90 % are shown).

Enzymic activities

The cells formed sulfide aerobically (weakly) and produced acid from glucose. Indole was not produced and the Voges–Proskauer test result was negative. There was active catalase, but test results for cytochrome oxidase and peroxidase reactions were negative. Nitrate was not reduced anaerobically. DNA was actively hydrolysed, but Tween-80, gelatin, starch and alginate were not.

Carbon metabolism

Strain IFAM EL-30^T grew well with 0·2 % (w/v) glucose, acetate, pyruvate, succinate, malate or glutamate. Weak growth was observed on citrate and on butyrate. Neither methanol nor methanesulfonic acid supported growth of this isolate. Metabolism of the following carbon sources occurred in the Biolog GP-System: acetate, propionate, pyruvate, 2′-deoxyadenosine, α-D-glucose, D-fructose, D-mannose, maltose, maltotriose, turanose, D-ribose, sucrose, D-mannitol, D-arabitol and dextrin. With the API 50CH System, the following carbon compounds were also metabolized: saccharose, trehalose and glycerol (weakly).

Chemotaxonomy

The isolate contained three phospholipids: diphosphatidylglycerol, phosphatidylglycerol and phosphatidylcholine. In addition, TLC revealed an unidentified glycolipid. The long-chain cellular fatty acids consisted of the following (%): i-14:0 (1·1), 14:0 (1·0), i-15:1 (1·0), i-15:0 (7·2), ai-15:0 (35·3), 16:1 (2·1), i-16:0 (15·9), 16:0 (4·3), i-17:0 (4·0) and ai-17:0 (23·0). Purified cell walls contained, besides muramic acid and glucosamine, the amino acids lysine, glutamic acid and alanine in a ratio of 1:2:2. From the chromatogram of the partial hydrolysate, the peptidoglycan type A4α, L-Lys-L-Glu was deduced. The DNA base ratio was determined as 66·1 mol % G+C.

16S rRNA gene sequence and phylogenetic analyses

The 16S rRNA gene sequence of isolate IFAM EL-30^T was determined by direct sequence analysis of PCR-amplified products. The sequence determined consisted of 1428 bases, and sequence searches of the EMBL database revealed *Nesterenkonia halobia* as the nearest relative of the isolate (96·2 % similarity). A tree depicting the phylogenetic relationships of IFAM EL-30^T and its closest relatives is shown in Fig. 2. The isolate formed a distinct subline with *Nesterenkonia halobia* within the grouping of *Kocuria* and *Rothia* species. The association of the novel isolate with *Nesterenkonia halobia* was statistically significant and was supported by a bootstrap resampling value of 100 %. All major groupings were confirmed using the parsimony program (data not shown). It is evident from the genotypic data that the isolate recovered from Ekho Lake represents a hitherto unknown species. Phylogenetically, the organism shows a significant association with *Nesterenkonia halobia* and clearly merits classification in the genus *Nesterenkonia*. A 16S rRNA gene sequence divergence of 3·8 % to *Nesterenkonia halobia*, together with morphological, biochemical and chemical differences, unequivocally demonstrates that the novel isolate represents a distinct species. Therefore, on the basis of the findings presented, we propose the isolate be classified in the genus *Nesterenkonia*. Characteristics which distinguish *Nesterenkonia lacusekhoensis* from its nearest relatives are shown in Tables 1 and 2.

Emended description of the genus *Nesterenkonia*

The description of the genus *Nesterenkonia* is as given by Stackebrandt *et al.* (1995) but with the following amendments: cells may consist of short rods sometimes showing branching, or of cocci. The peptidoglycan type is A4α, L-Lys-Gly-L-Glu or L-Lys-L-Glu. Menaquinones are present, MK-7, MK-8 and MK-9 predominating. The G+C content of the DNA is 66–72 mol %.

Table 1. Differential characteristics of isolate IFAM EL-30^T and the following related Gram-positive species: *Nesterenkonia halobia*, *Rothia mucilaginosa*, *Rothia dentocariosa*, *Rothia nasimurium*, *Kocuria kristinae*, *Kocuria varians*, *Kocuria rhizophila*, *Kocuria palustris* and *Kocuria rosea*

All of these bacteria can occur as cocci that may be clustered together. Abbreviations: w, weak reaction; v, variable; S, sensitive; R, resistant; NT, not tested, or information lacking. References: 1, Stackebrandt *et al.* (1995); 2, Onishi & Kamekura (1972); 3, Bergan & Kocur (1982); 4, Collins *et al.* (2000); 5, Georg & Brown (1967); 6, Schumann *et al.* (1999); 7, Collins & Jones (1980); 8, Kovács *et al.* (1999); 9, Kloos *et al.* (1974); 10, Georg (1974).

Characteristic	IFAM EL-30^T	*N. halobia*	*R. mucilaginosa*	*R. dentocariosa*	*R. nasimurium*	*K. kristinae*	*K. varians*	*K. rhizophila*	*K. palustris*	*K. rosea*
Cocci or other morphology	Short rods, may be branched	Pairs, tetrads	Pairs, tetrads	Rods, s.t. sept. a. branch	Cocci, s.t. ovoid	Tetrads	Rarely single, tetrads	Pairs or tetrads	Pairs or tetrads	Pairs or tetrads
Cell size (μm)	0·8–0·9 × 1–1·3	0·8–1·5	0·9–1·3	1·0–5·0[10]	NT	0·7–1·1	0·9–1·5	1·0–1·5 (2·0)	1·0–1·5 (2·0)	1·0–1·5
Colony pigment	Bright yellow	Colourless	Whitish	Creamy white	Colourless	Pale cream, pale orange	Yellow	Yellow	Pale yellow	Pink or red
Oxygen requirement	Aerobic	Aerobic	Facultatively anaerobic	Aerobic/microaerophilic	Facultatively anaerobic	Facultatively anaerobic	Aerobic	Aerobic	Aerobic	Aerobic
Sulfide formed	w	−	−	+	NT	−	−	w	w	−
Temp. optimum (°C)	27–33·5	NT	30–37	35–37	37	25–37	22–37	28	28	25–37
NaCl tolerance (%)	0 to > 15	5·0 to > 23	< 5·0	NT	NT	0 to < 15	< 10	> 15	< 7	< 7·5
pH tolerance	7·5–9·5	< 6·0–10·0	NT	5·4–8·8	NT	NT	NT	> 4·5–8·0	> 4·5–8·0	NT
Nitrate reduced anaerobically	−	−	+	+	+	−	+/−	−	+	+
Gelatin liquefied	−	−	+	+/−	NT	−/w	+/v	+	−	−
Citrate utilized	w	−	−	NT	NT	−	+	+	−	−/V
Quinones (%)										
MK-7	60	< 10	> 50	> 50	NT	−	−	−	−	−
MK-8	40	> 50	< 10	< 10	NT	−	−	−	−	−
MK-7 (H)	−	−	−	−	NT	> 50	> 50	51	87	< 10
MK-8 (H_2)	−	−	−	−	NT	> 10	< 10	39	2	> 50
Cell wall: interpeptide bridge	L-Lys-L-Glu	L-Lys-Gly-L-Glu	L-Lys-L-Ala	L-Lys-L-Ala	NT	L-Lys-L-Ala 3-4	L-Lys-L-Ala 3-4	L-Lys-Ala 3	L-Lys-Ala 3	L-Lys-L-Ala 3-4
G+C content (mol%)	66·1	71·5	59	65·4–69·7 (T_m)	56	67 (T_m)	66–72 (T_m)	69·4	69·6	66–75 (T_m)
Major fatty acids	ai-15:0, i-16:0, ai-17:0	ai-15:0; ai-17:0	ai-15:0, i-16:0	ai-15:0, i-16:0, ai-17:0	ai-15:0	ai-15:0; i-16:0	ai-15:0	ai-15:0, ai-17:0	ai-15:0, ai-17:0	ai-15:0
Utilization of:										
D-Fructose	+	NT	+	NT	NT	−	−	+	−	
D-Mannose	+	−	+	NT	NT	+	−/+	+	−	
Trehalose	+	−	+	+		+	−	−	−	
Origin	Ekho Lake, 23 m	Unrefined solar salt	Human mouth and respiratory tract	Human oral cavity	Healthy mouse (nose)	Human skin	Mammalian skin, soil, water	Typha rhizosphere	Typha rhizosphere	Soil, water
Type strain	DSM 12544^T (= CIP 107030^T)	DSM 20541 (= ATCC 21727)	DSM 20746 (= ATCC 25296)	DSM 46363 (= ATCC 17931)	CCUG 35957	DSM 20032 (= ATCC 27570)	DSM 20033 (= ATCC 15306)	DSM 11926	DSM 11925	DSM 20447 (= ATCC 186)
References		1, 2, 7	1, 3, 4	1, 3, 4, 5, 10	4	1, 8, 9	1, 8	8	8	1, 6

Table 2. Differential characteristics of *Nesterenkonia lacusekhoensis* IFAM EL-30^T and comparison with *Nesterenkonia halobia* DSM-20541^T

w, Weak reaction.

Characteristic	IFAM EL-30^T	*N. halobia*
Morphology	Short rods, occasionally branching, and cocci	Cocci in pairs or tetrads
Colony pigmentation	Bright yellow	Colourless
Temp. range for growth (°C)	8·5 to > 42	20–40
NaCl tolerance for growth (%)	0 to > 15	5·0 to > 23
Nicotinic acid stimulation	+	−
Starch hydrolysis	−	+
Sulfide formation	w	−
Acetoin formation	−	+
Oxidase activity	−	+
Phospholipids		
Phosphatidylcholine	+	−
Phosphatidylinositol	−	+
Menaquinones (%):		
MK-6	−	+
MK-7	60	< 10
MK-8	40	> 50
MK-9	−	> 10
Cell wall: interpeptide bridge	L-Lys-L-Glu	L-Lys-Gly-L-Glu
Major fatty acids (%):		
i-15:0	7·2	3·9
ai-15:0	35·3	65·0
i-16:0	15·9	6·3
ai-17:0	23·0	22·6
Utilization of trehalose	+	−
Citrate utilization	w	−
Acid from:		
Mannitol	−	+
Xylose	−	+
Lactose	−	+
Trehalose	+	−
Galactose	−	w
DNA G+C content (mol%)	66·1	71·5

Description of *Nesterenkonia lacusekhoensis* sp. nov.

Nesterenkonia lacusekhoensis (la.cus.ek.ho.en′sis N.L. fem. adj. *lacusekhoensis* 'of Ekho Lake', the lake in Antarctica from which the organism was isolated).

Cells consist of short rods with a mean size of 0·8 × 1·2 μm; sometimes branching is observed. In older cultures, cocci predominate. Cells are non-motile and do not contain poly-*β*-hydroxybutyrate granules. Colonies show a bright yellow pigmentation. Thiamin.HCl is required, and nicotinic acid and biotin stimulate growth. The optimal temperature for growth is 27–33·5 °C, and the optimal pH is 8·6. DNA is hydrolysed, but Tween 80, starch and alginate are not. Cells form sulfide but not indole. Methyl-red-negative and Voges–Proskauer-negative. Nitrate is not reduced anaerobically. Metabolizes sugars, sugar alcohols and some organic acids. Respiratory quinones are present with MK-7 (60%) and MK-8 (40%) predominating. The major fatty acids are ai-15:0, ai-17:0 and i-16:0. The peptidoglycan type is A4*α*, L-Lys-L-Glu. The G+C content of the DNA is 66·1 mol%. Habitat: hypersaline, heliothermal and meromictic Ekho Lake (23 m) in East Antarctica. Type strain: IFAM EL-30^T (DSM 12544^T = CIP 107030^T). The EMBL accession number for the 16S rRNA gene sequence of the type strain is AJ290397.

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