Nitriliruptor alkaliphilus gen. nov., sp. nov., a deep-lineage haloalkaliphilic actinobacterium from soda lakes capable of growth on aliphatic nitriles, and proposal of Nitriliruptoraceae fam. nov. and Nitriliruptorales ord. nov.

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A novel bacterial strain, designated ANL-iso2T, was obtained from an enrichment culture inoculated with a mixture of soda lake sediments by using isobutyronitrile (iBN) as the carbon, energy and nitrogen source at pH 10. The enrichment resulted in a stable binary culture containing iBN-degrading Gram-positive rods and a satellite Gram-negative gammaproteobacterium Marinospirillum sp. strain (ANL-isoa) scavenging the products of nitrile hydrolysis. Cells of the iBN-degrading strain, ANL-iso2T, were short, non-motile, non-sporo-forming rods. Strain ANL-iso2T was capable of utilizing propionitrile (C3), butyronitrile (C4), isobutyronitrile (C4), valeronitrile (C5) and capronitrile (C6) as the only growth substrate. Growth on nitriles was biphasic with fast initial hydrolysis of nitriles to the corresponding amides, carboxylic acids and ammonia and slow further utilization of these products resulting in biomass growth. Cells of strain ANL-iso2T grown with iBN were capable of extremely active hydration of a wide range of nitriles into the corresponding amides and much slower hydrolysis of these amides to the corresponding carboxylic acids. This indicated the presence of the nitrile hydratase/amidase pathway of nitrile degradation in the novel bacterium. Strain ANL-iso2T showed obligately alkaliphilic growth on iBN within the pH range 8.4–10.6, with optimum growth at 9.0–9.5. It was moderately salt-tolerant, with a salt range for growth of 0.1–2.0 M Na+ and an optimum salt concentration for growth of 0.2–0.3 M. The dominant fatty acids in the polar lipids were C16:0, iso-C14:0, C14:0, iso-C16:0 and C16:1ω7. The cell wall contained meso-diaminopimelic acid as the diagnostic diamino acid. Phylogenetic analysis placed strain ANL-iso2T within the class Actinobacteria as an independent lineage with only uncultured bacteria from soda lakes as its nearest relatives. On the basis of its unique phenotype and distinct phylogeny, strain ANL-iso2T is considered to represent a novel species of a new genus, for which the name Nitriliruptor alkaliphilus gen. nov., sp. nov. is proposed. The type strain of the type species, Nitriliruptor alkaliphilus, is ANL-iso2T (=DSM 45188T=NCIB 100119T=UNIQUEM U239T). Phylogenetic data suggest that the novel bacterium forms the basis of a new family Nitriliruptoraceae fam. nov. and a novel order Nitriliruptorales ord. nov. within the class Actinobacteria.

Abbreviations: DAP, diaminopimelic acid; iBN, isobutyronitrile.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ANL-iso2T is EF422408.

Organic compounds containing a C≡N bond (nitriles) are important intermediates and building blocks in the synthesis of organic compounds. There are also a few
examples of naturally occurring nitriles, which are either formed by cyanogenic plants from cyanide (Vetter, 2000) or produced during anaerobic degradation of amino acids (Harper & Gibbs, 1979). Most nitriles are toxic and difficult to degrade. Nevertheless, many bacterial species and some fungi can hydrolyse the nitrile bond via two different enzymic pathways (nitrile hydratase and nitrilase) with the corresponding carboxylic acid and ammonia as final products. Microorganisms possessing these enzymes are valuable biocatalysts and can be used either in organic synthesis or in environmental biotechnology (Kobayashi & Shimizu, 2000; Banerjee et al., 2002). Previously, all known nitrile-degrading microorganisms were neutrophilic, i.e. growing optimally at neutral pH values and in non-saline media.

We recently described the first example of a bacterium from soda lake sediments, *Natronocella acetonitrilica*, capable of growth with acetonitrile and propionitrile as carbon, energy and nitrogen source at haloalkaline conditions (Sorokin et al., 2007a). Furthermore, we also demonstrated the possibility of biodegradation of more complex nitriles, such as isobutyronitrile (iBN) [(CH$_3$)$_2$CHCN], by two haloalkaliphilic consortia, enriched from soda lakes and soda soils (Sorokin et al., 2007b). In the soda lake consortium, the key reaction in iBN hydrolysis was performed by the novel haloalkaliphilic actinobacterium described in the present paper. This bacterium is considered to represent a novel, deeply rooted cluster within the class *Actinobacteria*.

The isolation of an iBN-degrading consortium from soda lake sediments at pH 10 and the growth and metabolic properties of its members have been described previously (Sorokin et al., 2007b). A stable co-culture enriched from soda lake sediments capable of complete utilization of iBN at pH 10 and 0.6 M total Na$^+$ consisted of a motile spirillum as the dominant morphotype and short, non-motile rods. The spirillum was isolated from the mixed culture by using isobutyroamide, which is the first product of iBN hydrolysis in the nitrile hydrolase/amidase pathway. The organism was identified as a member of the genus *Marinospirillum* in the class *Gammaproteobacteria* (Sorokin et al., 2007b). Further experiments showed that this bacterium played the role of scavenging of iBN hydrolysis products released by the rod-shaped phenotype. The latter was obtained in pure culture by using high concentrations of iBN (up to 20 mM), which proved toxic for the *Marinospirillum* satellite organism. The iBN-hydrolysing strain was designated ANL-iso2$^T$.

Phase-contrast photomicrographs were obtained by using a Zeiss Axioplan Imaging 2 microscope. For electron microscopy, cells were fixed with glutaraldehyde (final concentration 3 %, v/v) and positively contrasted with 1 % (w/v) uranyl acetate. For thin sectioning, cells were fixed in 1 % (w/v) OsO$_4$ + 0.5 M NaCl for 3 h at room temperature, washed and stained overnight with 1 % (w/v) uranyl acetate, dehydrated in an ethanol series and embedded in Epon resin. Thin sections were stained with 1 % (w/v) lead citrate. Fatty acids of the cellular lipids were extracted with a mixture of methanol/chloroform and analysed by GC-MS according to Zhilina et al. (1997). The cell-wall fraction was obtained and analysed according to Streshinskaya et al. (1979). A cell suspension in 1 % (w/v) SDS was disrupted by sonication and heated briefly (100 °C, 5 min). Cell walls were separated by fractional centrifugation and were purified by using trichloroacetic acid and trypsin. Quantitative determination of the cell-wall amino acids was performed with an LC 600 E amino acid analyser (Biotronic) after acid hydrolysis (6 M HCl, 100 °C, 18 h). Isomers of diaminopimelic acid (DAP) were determined by TLC on cellulose (modified method of Hasegawa et al., 1983).

Strain ANL-iso2$^T$ formed flat, spreading microcolonies on the iBN alkaline agar after 1 month of incubation. Cells were small, non-motile rods, occurring singly or in pairs under most conditions (Fig. 1a). When grown on glucose and yeast extract, chain formation was observed (Fig. 1b). The cell wall was of the Gram-positive type (Fig. 1c). The cell wall contained alanine, glutamic acid and meso-DAP in a molar ratio of 2:1:1, which indicated the presence of the A$_2$-type peptidoglycan (Schleifer & Kandler, 1972). Small amounts (up to a molar ratio of 0.4) of other amino acids were also detected, which suggested the presence of additional peptides. Furthermore, glucose, galactose and glyceral were found in the cell-wall hydrolysates (hydrolysis at 2 M HCl, 3 h, 100 °C). These data, along with the presence of glycerol phosphates, phosphodiesters and an unidentified amino sugar, as revealed by electrophoresis of the cell-wall hydrolysates, suggested the presence of an anionic carbohydrate-containing polymer(s) in the cell wall of strain ANL-iso2$^T$ (Naumova et al., 2001). Analysis of whole-cell fatty acids of strain ANL-iso2$^T$ grown with iBN at pH 10, 0.6 M Na$^+$ and 30 °C demonstrated a dominance of saturated components, including C$_{16:0}$ (19 %), iso-C$_{14}$ (17 %), C$_{14:0}$ (13 %) and iso-C$_{16}$ (8 %). Unsaturated components were represented by C$_{16:1}$-o7 (16 %), C$_{17:1}$-o8 (5 %) and C$_{18:1}$-o9 (4 %).

Strain ANL-iso2$^T$ completely degraded iBN, utilizing it as a sole source of energy, carbon and nitrogen. However, growth of the pure culture was very slow, not only with nitriles but also with other growth substrates in general. It converted iBN rapidly into isobutyroamide, isobutyrate and ammonia during the initial phase with little biomass growth. Subsequent actual biomass growth was very slow, with $\mu_{\text{max}}$=0.035 h$^{-1}$. Strain ANL-iso2$^T$ was also capable (after adaptation) of growth on a range of linear aliphatic nitriles with different chain lengths, such as propionitrile (C$_3$), butyronitrile (C$_4$), valeronitrile (C$_5$) and capronitrile (C$_6$), as the carbon, energy and nitrogen source, with $\mu_{\text{max}}$ values similar to that with iBN. However, it was not able to grow on acetonitrile (C$_2$). Other compounds utilized as single carbon and energy source included the respective organic acids produced from the aliphatic nitriles listed above, acetate, malate, fumarate, succinate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, pyruvate, and pyruvate.
citrate, D-glucose, D-fructose, maltose, D-mannose, melezitose, \(\alpha\)-\(\alpha\)-trehalose, sucrose, D-arabinose, inositol, cellobiose and glycerol. Growth was also possible on complex media, including yeast extract or peptone broth, but was much slower than in the presence of organic acids or sugars. Anaerobic fermentative growth with glucose, fructose or iBN plus nitrate was not observed. Strain ANL-iso2\(^T\) was able to hydrolyse and utilize gelatin, but not starch, casein, cellulose, chitin, pectin or xylan.

The pH range for growth of strain ANL-iso2\(^T\) at 0.6 M Na\(^+\) with iBN was 8.4–10.4 (optimum growth at pH 9.0–9.5) and the salt range for growth at pH 10 was 0.1–2.0 M total Na\(^+\) (optimum growth at 0.2–0.3 M), qualifying the organism as a moderately salt-tolerant, obligate alkaliphile (Fig. 2). In contrast to the conditions for growth, hydrolysis of iBN by washed cells and cell-free extract was possible at acidic to neutral pH with an optimum at pH 8, indicating intracellular localization of the nitrile hydratase/amidase system (Fig. 2). The metabolic potential of strain ANL-iso2\(^T\) was not limited by the ability to hydrolyse C\(_3\)–C\(_6\) aliphatic nitriles, which are utilized as growth substrates. Experiments with washed cells grown with iBN demonstrated that the actual range of nitriles being converted was much broader than utilized for growth, including such industrially important nitriles as...
acrylonitrile, metacrylonitrile, acetonitrile, benzonitrile, nicotinonitrile and adiponitrile, but also more industrially challenging nitriles such as tertiary nitriles and \( \text{\( \alpha \)} \)-azidonitriles. In all cases, nitrile hydratase activity was two to three orders of magnitude higher than the corresponding amidase activity.

Isolation of DNA and subsequent determination of G+C content were performed according to Marmur (1961) and Marmur & Doty (1962), respectively. Isolation of template DNA, amplification of the 16S rRNA gene of strain ANL-iso2\(^T\) and its sequencing have been described previously (Sorokin et al., 2007a). The 16S rRNA gene sequence of strain ANL-iso2\(^T\) was aligned with those from GenBank by using CLUSTAL W. Phylogenetic trees were reconstructed via four different algorithms by using the TREECONW software package (Van de Peer & De Wachter, 1994). Pairwise evolutionary distances (expressed as estimated changes per 100 nt) were calculated by using the method of Jukes & Cantor (1969). Phylogenetic trees were then reconstructed by using three different algorithms. As the tree topology was very similar in all three cases, final results are presented for the neighbour-joining analysis only. Bootstrap analysis (based on 1000 replications) was used to validate the reproducibility of the branching patterns of the trees. Phylogenetic analysis based on 16S rRNA gene sequencing placed the nitrile-utilizing alkaliphile within the class Actinobacteria as a novel deep lineage (Fig. 3). It formed an independent cluster together with several uncultured actinobacteria from various habitats, including soda lakes in the USA, Egypt and Russia (Humayoun et al., 2003; Mesbah et al., 2007; Foti et al., 2008). However, strain ANL-iso2\(^T\) showed no more than 85% 16S rRNA gene sequence similarity to any recognized taxon. Therefore, the only safe conclusion that could be drawn from 16S rRNA gene sequence comparison was that the novel bacterium is a member of the class Actinobacteria. At the time of writing, the class Actinobacteria consists of six orders, among which the order Actinomycetales is the most numerous and diverse (see Fig. 3). Strain ANL-iso2\(^T\), despite being a typical actinobacterium in its morphotype, phylogenetically forms an independent lineage. The lack of such actinobacteria in culture might originate from their very slow growth and specialization on exotic toxic substrates such as nitriles. It is interesting to note that actinobacterial genera such as Rhodococcus, Arthrobacter, Nocardia and Gordonia contain many highly active and versatile nitrile-hydrolysing strains, including some that are industrially important (Bunch, 1998; Kobayashi & Shimizu, 2000; Brady et al., 2004).

Strain ANL-iso2\(^T\) can be regarded as the first haloalkaliphilic versatile nitrile degrader and as a representative of a novel deep phylogenetic lineage within the class Actinobacteria. Based on unique phenotypic properties and distinct phylogeny, we suggest that strain ANL-iso2\(^T\) represents a novel species of a new genus, for which the name Nitriliruptor alkaliphilus gen. nov., sp. nov. is proposed, and that this forms a novel family Nitriliruptoraceae fam. nov. and a new order Nitriliruptorales ord. nov. within the class Actinobacteria.

**Description of Nitriliruptor gen. nov.**


Gram-positive rods. Cell wall contains *meso*-DAP. The dominant fatty acids in the polar lipids are saturated C\(_{14}\)-C\(_{16}\) components. Aerobic, and utilize short-chain organic acids, amides and aliphatic nitriles as energy and carbon source. Alkaliphilic and moderately salt-tolerant. A member of the class Actinobacteria. The type species is *Nitriliruptor alkaliphilus*. Found in soda lakes.

**Description of Nitriliruptor alkaliphilus sp. nov.**


Cells are non-motile, 0.4 × 1–3.0 μm in size. Colonies are colourless, flat and spreading. The dominant cellular fatty acids are C\(_{14}:0\), C\(_{16}:0\) iso-C\(_{14}\)-iso-C\(_{16}\) and C\(_{16}:1\)\(\gamma\)7. Strictly aerobic. Oxidase- and catalase-positive. The cell-wall peptidoglycan is of A\(_{1}\)-type, containing *meso*-DAP. Able to grow with C\(_{2}\)-C\(_{6}\) aliphatic nitriles and corresponding amidases as energy, carbon and nitrogen source. Other growth substrates include simple organic acids (acetate, propionate, pyruvate, butyrate, isobutyrate, valerate, succinate, malate, citrate and fumarate), sugars and sugar alcohols (glucose, maltose, fructose, arabinose, mannose, sucrose, z,z-trehalose, melezitose, inositol and glycerol) and complex organic substrates such as yeast extract, peptone and gelatin. Hydrolytic capacity is absent. Able to metabolize (without growth) a large spectrum of aliphatic and some aromatic nitriles via the nitrile hydratase/amidase enzyme system. Obligately alkaliphilic with a pH range for growth between 8.2 and 10.6 (optimum at pH 9.0–9.5) and moderately salt-tolerant with a salt range for growth of 0.1–2.0 M Na\(^+\) (optimum at 0.2–0.3 M). Mesophilic with an optimum temperature for growth of 32 °C. The G+C content of the genomic DNA is 70.8 mol% (\(T_m\)).

The type strain, ANL-iso2\(^T\) (=DSM 45188\(^T\)=NCCB 100119\(^T\)=UNIQEM U239\(^T\)), was isolated from soda lake sediments of the Kulunda Steppe (Altai, Russia).

**Description of Nitriliruptoraceae fam. nov.**

*Nitriliruptoraceae* (Ni.tri.li.ru.p.to.ra’ceae. N.L. masc. n. *Nitriliruptor* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Nitriliruptoraceae* the family of the genus *Nitriliruptor*).

Gram-positive rods. Aerobic heterotrophs with the ability to degrade organic nitriles. Members are alkaliphilic and moderately salt-tolerant. A member of the order Nitriliruptorales in the class Actinobacteria. The type genus...

**Fig. 3.** Phylogenetic position of strain ANL-iso2\textsuperscript{T} and its uncultured relatives within the class *Actinobacteria* based on 16S rRNA gene sequence analysis. Tree topology and evolutionary distances were calculated by the neighbour-joining method with the corrections of Jukes & Cantor (1969). Numbers at nodes are bootstrap percentages for the clade of this group based on 1000 replications; only values above 50% are shown. Bar, 0.05 substitutions per nucleotide position.

**Description of *Nitriliruptorales* ord. nov.**

*Nitriliruptorales* (Ni.tri.li.rup.tor.a’les. N.L. masc. n. *Nitriliruptor* type genus of the order; -ales ending to denote an order; N.L. fem. pl. n. *Nitriliruptorales* the order of the genus *Nitriliruptor*).
Description is as for the family Nitriliruptoraceae. The 16S rRNA gene signature pattern is as that of the family Nitriliruptoraceae. The type genus is Nitriliruptor.

Acknowledgements

This work was supported by NWO-RFBR (grant 047.011.2004.010), RFBR (grant 07-04-00153) and by the Program on Molecular and Cell Biology RAS. We are grateful to E. Barinova for help with electron microscopy and to A. Lysenko for determination of the DNA G+C content.

References


