Anaerobacillus isosaccharinicus sp. nov., an alkaliphilic bacterium which degrades isosaccharinic acid

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Abstract

Strain NB2006\textsuperscript{T} was isolated from an isosaccharinate-degrading, nitrate-reducing enrichment culture in minimal freshwater medium at pH 10. Analysis of the 16S rRNA gene sequence indicated that this strain was most closely related to species of the newly established genus Anaerobacillus. This was supported by phenotypic and metabolic characterisation that showed that NB2006\textsuperscript{T} was rod-shaped, Gram-stain-positive, motile and formed endospores. It was an aerotolerant anaerobe and an obligate alkalophile that grew at pH 8.5–11, could tolerate up to 6 % (w/v) NaCl, and grew at a temperature between 10 and 40°C. In addition, it could utilise a number of organic substrates, and was able to reduce nitrate and arsenate. The predominant cellular fatty acids were C\textsubscript{16:0}, C\textsubscript{16:1ω11c}, anteiso-C\textsubscript{15:0}, iso-C\textsubscript{15:0}, C\textsubscript{16:1ω7c}/iso-C\textsubscript{15:0} 2-OH and C\textsubscript{14:0}. The cell wall peptidoglycan contained meso-diaminopimelic acid and the DNA G+C content was 37.7 mol%. In silico DNA–DNA hybridization with the four known species of the genus Anaerobacillus showed 21.8, 21.9, 22.4, and 21.5 % relatedness to Anaerobacillus arseniciselenatis DSM 15340\textsuperscript{T}, Anaerobacillus alkalidiazotrophicus DSM 22531\textsuperscript{T}, Anaerobacillus alkalilacustris DSM 18345\textsuperscript{T}, and Anaerobacillus macyae DSM 16346\textsuperscript{T}, respectively. NB2006\textsuperscript{T} differed from strains of other species of the genus Anaerobacillus in its ability to metabolise isosaccharinate, an alkaline hydrolysis product of cellulose. On the basis of the consensus of phylogenetic and phenotypic analyses, this strain represents a novel species of the genus Anaerobacillus, for which the name Anaerobacillus isosaccharinicus sp. nov. is proposed. The type strain is NB2006\textsuperscript{T} (=DSM 100644\textsuperscript{T}=LMG 30032\textsuperscript{T}).

The genus Anaerobacillus was proposed by Zavarzina et al. [1] to contain strictly anaerobic or aerotolerant, Gram-positive rods that are obligate or moderately alkaliphilic, halotolerant or moderately halophilic, grow through fermentation or anaerobic respiration and are able to reduce arsenate. At the time of writing, the genus Anaerobacillus comprises only four species with validly published names: Anaerobacillus macyae [2], Anaerobacillus alkalidiazotrophicus [3], Anaerobacillus arseniciselenatis [4], and Anaerobacillus alkalilacustris [1], with A. arseniciselenatis as the type species.

Isosaccharinate is a cellulose alcali hydrolysis product that is found in paper pulp effluent streams [5]. It is also expected to form under the anaerobic, hyperalkaline conditions of a geological disposal facility for intermediate-level radioactive waste [6], and has the potential to increase radionuclide mobility in the subsurface [7, 8]. For these reasons, the microbial degradation of isosaccharinate, has received considerable recent attention [9–11]. Two aerobic Gram-negative bacteria, capable of isosaccharinate degradation have been isolated previously [12, 13]. Here we describe a novel species belonging to the newly established genus Anaerobacillus that utilises isosaccharinate under anaerobic, alkaline conditions.

An isosaccharinate-degrading, nitrate-reducing enrichment culture grown on minimal freshwater medium at pH 10 had been set up previously and maintained at 20 °C under anaerobic conditions [14]. The freshwater medium contained per litre 2.5 g NaHCO\textsubscript{3}, 0.25 g NH\textsubscript{4}Cl, 0.6 g Na\textsubscript{2}HPO\textsubscript{4}, 0.1 g KCl, 2 g NaNO\textsubscript{3}, 0.8 g calcium isosaccharinate and 10 ml mineral and vitamin mixes [15], and was pH adjusted to pH 10 using NaOH, and made aerobically by flushing with N\textsubscript{2} for 1 h [14]. The enrichment culture was inoculated with 5 % (w/v) of a sediment sample from a legacy lime-kiln in Harpur Hill, Buxton, United Kingdom [16, 17], and was enriched for isosaccharinate-degrading bacteria at 20 °C by transferring 1 % inoculum into fresh medium every month [14]. At the end of the fourth subculture of this enrichment...
Culture 50 µl samples were plated on agar plates as described previously [14]. Only one colony morphology was observed, and this showed protruding, small, round and transparent colonies after 3 days of incubation under anaerobic conditions at 20 °C. Five representative colonies were selected as described previously [14], and were all found to degrade isosaccharinate under nitrate-reducing conditions at pH 10.

The 16S rRNA genes of these isolates, were amplified by PCR and sequenced using the following universal primers: 8F (5'-AGAGTTTGATCCTGGCTCA-3'), 530F (5'-GTGCAACCCCGCGGCCC-3') and 1492R (5'-TACGGYTACCTTACGACCTT'3') [18, 19], as described previously [14]. The almost complete (1520 bp) 16S rRNA gene sequences of these strains showed more than 99% similarity, indicating that they represented the same species, therefore, strain NB2006T was selected as a representative strain. Phylogenetic neighbours with validly published names based on the 16S rRNA gene sequence similarities were identified using the EZBioCloud identification service (http://www.ezbiocloud.net; [20]). The 16S rRNA gene sequence of NB2006T was aligned to available sequences of all species with validly published names belonging to the genus Anaerobacillus, and some related species of the genus Bacillus that showed more than 96% identity, using Clustal W [21] in the software package MEGA version 7 [22]. Phylogenetic trees were then reconstructed with the neighbour-joining algorithm [23] using maximum composite likelihood distance [24], and the maximum-likelihood algorithm using Kimura’s two-parameter model [25], with the complete deletion option, gamma distributed with an invariant sites value of 5, and bootstrap [26] of 1000 replications.

The results of phylogenetic analysis based on the 16S rRNA gene sequence indicated that NB2006T was most closely related to species of the newly established genus Anaerobacillus, A. macyae JMM-4T (97.96 %), A. alkalidiazotrophicus MS 6T (97.56 %), A. arseniciselenatis DSM 15340T (97.45 %) and A. alkalilactis Z-0521T (97.01 %). Both the neighbour-joining and maximum-likelihood phylogenetic trees revealed that NB2006T clustered with other members of the genus Anaerobacillus with a bootstrap value of 99 %, and clearly separated from the aerobic, alkaliphilic bacilli (Fig. S1, available in the online version of this article).

NB2006T was characterised using a polyphasic taxonomic approach and compared with reference strains of species of the genus Anaerobacillus obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection (Braunschweig, Germany): A. arseniciselenatis DSM 15340T (=EAI H1T=ATCC 700614T=KCTC 5192T), A. alkalidiazotrophicus DSM 22531T (=MS 6T=NCCB 100213T=UNIQEM U377T) and A. alkalilactis DSM-18345T (=Z-0521T=VKM B-2403T). These strains grew well in the same medium as NB2006T (for A. arseniciselenatis DSM 15340T, 0.9 % NaCl was added). A. macyae DSM 16346T (=JMM-4T=CIP 108766T=JCM 12340T) did not grow in the same medium, therefore, it was not included in the phenotypic analysis.

NB2006T was maintained in a liquid medium containing, per litre, 5 g Na2CO3, 2.5 g NaHCO3, 0.25 g NH4Cl, 0.6 g NaH2PO4•H2O, 0.1 g KCl, 2 g NaNO3, 3 g yeast extract and 10 ml of the same mineral mix [15], at pH 9.8 and 30 °C. It was also maintained at 4 °C on solid agar plates containing the same medium, or in medium with 20 % (v/v) glycerol at −80 °C. Colony appearance was examined after incubation on solid medium at 30 °C for 3 days. Growth at different pH values (pH 7.5–11.5, at intervals of 0.5 pH units, adjusted by varying the ratio of NaHCO3 and Na2CO3), temperatures (4, 10, 20, 30, 35, 40 °C), NaCl (w/v) concentrations (0, 0.5, 1.0, 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 9.0 and 12.0 %) and NaHCO3 (w/v) concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 %) was tested by measuring the increase in OD600 after incubation of liquid cultures for 3 days at 30 °C (except for the temperature test). The relationship to oxygen was assessed by observing bacterial growth in vertical agar tubes containing resazurin solution, and by observing bacterial growth in liquid cultures flushed with different ratios of O2:N2 (0–20 % O2, in 5 % increments). The morphology of the cells and spores was examined by light and electron microscopy. Gram-staining was done on heat-fixed slides using a Gram-staining kit (Sigma). Flagella were stained on air-dried slides using Flagella Stain Dippers (BD). Endospores were stained on heat-fixed slides with malachite green (Pro-Lab) over steam for 5 min and then counterstained with Safranin T solution (Fluka) for 1 min. Motility was observed in wet mounts and by growth on semisolid culture medium (by adding 0.1 % (w/v) agar). Catalase activity was determined by production of bubbles after the addition of a drop of 3 % (v/v) H2O2. Oxidase activity was determined by the oxidation of tetramethyl-p-phenylenediamine. Casein, gelatin and starch hydrolysis were tested on skimmed milk agar plates (Sigma), by checking for discoloration of the media, gelatin tubes (Sigma), by checking for solidification of the media at 4 °C, and soluble starch agar plates (Fisher), by checking for colouration after the addition of Gram’s iodine solution, incubated for 3 days at 30 °C. All experiments were prepared in duplicate, and the reference strains were tested alongside the novel isolate (NB2006T).

The utilisation of different electron donors and acceptors was tested in 10 ml butyl-stoppered serum bottles containing the same anaerobic freshwater minimal medium, and measuring the OD600 at the beginning of the experiment, and after 3 days of incubation at 30 °C. The concentrations of the carboxylic acids, nitrate, nitrite, selenate and selenite were measured by ion-exchange chromatography as described previously [14]. The concentrations of arsenate and arsenite in solution were determined by coupled ion chromatography-inductively coupled plasma-mass spectrometry using a method developed previously [27]. For the electron donor tests, the yeast extract was removed from the growth medium and 5 mM of the Na salt of carboxylic acids...
(formate, acetate, lactate, pyruvate, succinate, malate, fumarate, gluconate, isosaccharinate, citrate, malonate), sugars and sugar alcohols (glucose, fructose, sucrose, lactose, sorbitol, mannitol, myo-inositol) and amino acids (glycine, serine, cysteine, glutamate, histidine, asparagine, arginine) were added. For the utilisation of electron acceptor tests, nitrate was removed and the medium was supplemented with 10 mM Na$_2$SO$_4$, 2 mM Na$_2$SeO$_4$ or 2 mM Na$_2$AsO$_4$. All experiments were prepared in duplicate, and the reference strains were tested alongside NB2006$^T$.

Colonies were protruding, small, round and transparent after 3 days of incubation under anaerobic conditions at 30 °C. Growth was observed after 3 days of incubation at pH values between 8.5 and 11, with optimal growth at pH 9.8–10. Temperatures between 10 and 40 °C were tested alongside NB2006$^T$ showed growth in the anaerobic zone in vertical agar tubes and grew in liquid cultures flushed with O$_2$. Light and electron microscopy revealed that NB2006$^T$ cells are Gram-stain-positive rods, that are 2–5×0.5–0.7 µm in size (Fig. S2), motile using four peritricus flagella, can live singly or in chains of up to six connected rods and can form circular, subterminal spores. NB2006$^T$ showed growth in the anaerobic zone in vertical agar tubes and grew in liquid cultures flushed with O$_2$. Light and electron microscopy revealed that NB2006$^T$ cells are Gram-stain-positive rods, that are 2–5×0.5–0.7 µm in size (Fig. S2), motile using four peritricus flagella, can live singly or in chains of up to six connected rods and can form circular, subterminal spores. NB2006$^T$ was catalase- and oxidase-positive, hydrolysed gelatin and starch and was able to utilise lactate, pyruvate, succinate, malate, fumarate, gluconate, isosaccharinate, glucose, fructose, sucrose, serine, asparagine and arginine as electron donors. It grew by anaerobic respiration, where it reduced nitrate to nitrite and arsenate to arsenite. It also grew by fermentation of yeast extract or gluconate, which led to the production of acetate and formate as fermentation products. A phenotypic and biochemical comparison between NB2006$^T$ and the selected type strains of species of the genus *Anaerobacillus* showed metabolic differences between the tested strains (Table S1).

Cellular fatty acid composition, cell-wall peptidoglycan analysis, and determination of the DNA G+C content of NB2006$^T$ were performed by the DSMZ Identification Service (Braunschweig, Germany).

The predominant cellular fatty acids (>5 %) of NB2006$^T$ were C$_{16:0}$ (23.35 %), C$_{16:1}$ω11c (22.96 %), anteiso-C$_{15:0}$ (15.33 %), iso-C$_{15:0}$ (8.30 %), C$_{16:1}$ω7c/iso-C$_{15:0}$2-OH (6.28 %), and C$_{14:0}$ (5.48 %) which is consistent with other members of the genus *Anaerobacillus* [1, 3]. The cell-wall diagnostic diamino acid was meso-diaminopimelic acid, and the peptidoglycan type was A1γ. The DNA G+C content of NB2006$^T$, determined by HPLC, was 37.7 mol%.

Draft genomes of the species of the genus *Anaerobacillus* used in this study have been published recently [28, 29]. The genome sequences with accession numbers MLQK00000000, MLQS00000000, MLQR00000000, LQXD00000000, and LEK00000000 were retrieved from the NCBI database. Original average nucleotide identity, and average nucleotide identity of orthologous genes were calculated using the Orthologous Average Nucleotide Identity Tool version 0.93 [30]. Digital DNA–DNA hybridization values were determined using the Genome-to-Genome Distance Calculator version 2.1 using formula 2 [31]. The genomes were re-annotated using Prokka version 1.11 [32], and the core genome that contains the genes shared by all species of the genus *Anaerobacillus* was identified using the software package Roary version 3.8.0 [33], with a minimum sequence identity of 70 % [34]. A phylogenetic tree was reconstructed using the neighbour-joining algorithm [23] with maximum composite likelihood distance [24], and the maximum-likelihood

**Fig. 1.** Neighbour-joining phylogenetic tree reconstructed from the core genes (281 genes) of species closely related to NB2006$^T$. Bootstrap values >50 %, based on 1000 replicates, are indicated on branch points. *Bacillus subtilis* was used as an outgroup. Bar, 0.05 nucleotide substitutions per site. A maximum-likelihood phylogenetic tree showed the same results as the neighbour-joining phylogenetic tree.
algorithm using Kimura’s two-parameter model [25] and a
gamma correction value of 0.5 using MEGA version 7 [22].
Functional annotation was performed based on the Kyoto
Encyclopedia of Genes and Genomes (KEGG) database
(http://www.genome.jp/kegg/), and metabolic pathways for
several phenotypic features were mapped using KEGG Mapper
(http://www.genome.jp/kegg/). Key enzymes were identified
from the metabolic pathway graphs (Figs S3–S12), and the presence of genes coding for these enzymes in the tested
genome was determined (Table S5).

The genome of NB2006T was 4.95 Mbp, and contained 4984
genomes [29]. It is important to note that the calculated DNA
G+C content was slightly lower (35.8 mol%) than that
acquired by direct HPLC analysis (Table S2), which may be
due to the genome sequence being incomplete [29]. The
original average nucleotide identity, and the average nucleo-
tide identity of orthologous genes between NB2006 and
the other species of the genus Anaerobacillus were 66.60–
74.92 %, and 67.24–75.62 % (Table S3), respectively, well
below the threshold of 95–96 % for species delineation [35, 36]. Furthermore, the digital DNA–DNA hybridization values
were 21.5–22.4 % (Table S3), well below the threshold of
70 % for species delineation [35]. Phylogenetic analysis of
the core genes (294 genes) showed a different tree topology
than that based on the 16S rRNA gene sequence, which is
more consistent with phenotypic observations; NB2006 clus-
tered more closely with the alkaliphilic species of the
Anaerobacillus genus (A. arseniciselenatis, A. alkalilacustris,
and A. alkalidiazotrophicus), rather than with the slightly
alkalitolerant species (A. maccyae). The strictly fermentative
species (A. alkalilacustris, and A. alkalidiazotrophicus) also clus-
tered together with this analysis (Fig. 1).

Genomic comparisons revealed that NB2006T shared 294
genomes with all other members of the genus Anaerobacillus
(core genomes), and it shared 1745 genes with at least one spe-
cies of the genus (accessory genomes), while 2932 genes were
unique to NB2006T (Table S4). Generally, the metabolic
functions that were identified from the genome (Table 1)
agreed well with those observed in the growth experiments
(Table S1), except in the dissimilatory nitrate reduction
pathway, where the A. alkalilacustris DSM 18345T genome
codes for the periplasmic nitrate reductase napA (Table S5, Fig. S11), but the bacterium does not reduce nitrate; and in
the sorbitol utilisation pathway, where the A. alkalidiazotro-
phicus DSM 22531T genome does not code for the L-iditol
2-dehydrogenase gene (Table S5 and Fig. S5), but the bio-
chemical tests indicated that it can degrade sorbitol. It is
possible that these inconsistencies are due to the incomplete
nature of the genomes being compared, and may be resolved
when complete genomes are sequenced and annotated.

Based on the consensus of phylogenetic and phenotypic
analyses, strain NB2006T represents a novel species of the
genus Anaerobacillus, for which the name Anaerobacillus
isosaccharinicus sp. nov. is proposed. The ability to utilise
isosaccharinic acid has not been demonstrated previously
for this genus, and indeed has not, to our knowledge, been
demonstrated under anaerobic conditions for any isolate in
pure culture before.

Table 1. Comparison of phenotypic features captured from the
genomes of A. isosaccharinicus NB2006T and related taxa

<table>
<thead>
<tr>
<th>Strains</th>
<th>A. isosaccharinicus NB2006T</th>
<th>A. arseniciselenatis DSM 15340T</th>
<th>A. alkalidiazotrophicus DSM 22531T</th>
<th>A. alkalilacustris DSM 18345T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Catalase*</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Oxidase*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Reduction of Nitrate†</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>4</td>
<td>Sulfate†</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>5</td>
<td>Utilisation of Fructose†</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>6</td>
<td>Galactose†</td>
<td>–</td>
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<td>Sucrose†</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>8</td>
<td>Cellobiose‡</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>9</td>
<td>Trehalose</td>
<td>–</td>
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<td>+</td>
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<td>10</td>
<td>Arabinose</td>
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<tr>
<td>11</td>
<td>Mannose</td>
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<td>12</td>
<td>Xylose</td>
<td>+</td>
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<tr>
<td>13</td>
<td>L-Leucine</td>
<td>–</td>
<td>–</td>
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<tr>
<td>14</td>
<td>myo-Inositol §</td>
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<tr>
<td>15</td>
<td>Production of Indole</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>Acetoin (Voges–Proskauer test)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*a*Represents features that show agreement between the observed
phenotype and that captured from the genomes.

†Represents features that show differences between the observed
phenotype and that captured from the genomes.

**DESCRIPTION OF ANAEROBACILLUS ISOSACCHARINICUS SP. NOV.**

*Anaerobacillus isosaccharinicus* (i.so.sac.cha.ri’ni.cus. N.L. neut. n. *acidum* isosaccharinicum isosaccharinic acid; N.L. masc. adj. isosaccharinicus pertaining to isosaccharinic acid).

Cells are Gram-stain-positive, aerotolerant anaerobes,
motile, using four peritrichous flagella, rods of 0.5–0.7 µm
diameter, and 2–5 µm length and form circular subterminal
endospores with unswollen sporangia. Colonies were
producing, small, round and transparent after 3 days of incubation
under anaerobic conditions at 30 °C. Bacterial growth
was observed at pH values between 8.5 and 11 (optimal at
pH 9.8–10), temperatures between 10 and 40 °C (optimal at
30 °C), 0–6.0 % (w/v) NaCl (optimal at 2.0 %) and 0–5.0 %
(w/v) NaHCO3 (optimal at 0.5 %). Cells were catalase-
and oxidase-positive. The cell-wall peptidoglycan contains
meso-diaminopimelic acid. The primary fatty acids were C16:0-
The type strain is NB2006\(^T\) (=DSM 100644\(^T\) =LMG 30032\(^T\)), which was isolated from a legacy limekiln effluent in Harpur Hill, Buxton, UK. The DNA G+C content of the type strain is 37.7 mol%.

### References

1. Zavarzina DG, Tourova TP, Kolganova TV, Bouligyina ES, Zhilina TN. Description of Anaerobacillus alkalilacustre gen. nov., sp. nov. —Strictly anaerobic diazotrophic bacillus isolated from soda lake and transfer of Bacillus arsenicilenatis, Bacillus macyae, and Bacillus alkalidiazotrophicus to Anaerobacillus as the new combinations A. arsenicilenatis comb. nov., A. macyae comb. nov., and A. alkalidiazotrophicus comb. nov. Microbiology 2009;78:723–731.


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