Anaerobranca zavarzinii sp. nov., an anaerobic, alkalithermophilic bacterium isolated from Kamchatka thermal fields

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A novel obligately anaerobic, alkalithermophilic, chemo-organotrophic bacterium was isolated from a small and very shallow geothermally heated pool at Pushino (Kamchatka, Far East Russia). The bacterium, designated strain JW/VK-KS5YT, was a Gram staining negative, Gram type positive rod. The cells were sometimes branched, with a tendency to grow in long chains, and were non-spore-forming and non-motile. The shortest observed doubling time was 28 min when the novel strain was grown at 54–60 °C in 120 mM sodium carbonate-containing medium at pH 8.5–9.0. The novel bacterium grew on yeast extract and soytone as sole carbon and energy sources but could also use fumarate, thiosulfate and sulfur as electron acceptors. The DNA G+C content was 32.5 mol%. Based on phylogenetic, DNA–DNA hybridization and phenotypic data, it was concluded that isolate JW/VK-KS5YT (=VKM B-2436T=DSM 18970T) represents the type strain of a novel species, Anaerobranca zavarzinii sp. nov.

Alkalithermophilic bacteria are of interest due to their ability to live in extreme environments. Being double extremophiles, alkalithermophiles are potential sources for novel enzymes as well as being model organisms for studying the origin of life and life on early Earth (Wiegel, 1998; Wiegel & Adams, 1998; Kevbrin et al., 2004). To date, the genus Anaerobranca (Firmicutes branch) is an example of a genus consisting entirely of anaerobic, alkalithermophilic species: Anaerobranca horikoshii (Engle et al., 1995), Anaerobranca gottschalkii (Prowe & Antranikian, 2001) and Anaerobranca californiensis (Gorlenko et al., 2004).

The aim of our study of the hot springs of the Kamchatka peninsula was to investigate bacteria that were able to grow simultaneously at alkaline pH25 ~8.0 and at 60 °C and hence fell within our definition of alkali thermophiles (Wiegel, 1998). Strain JW/VK-KS5YT was isolated from a 30 ml enrichment inoculated with 2 ml of a combined water–sediment slurry sample collected at Pushino (Kamchatka, Far East Russia) in August 2000. The sample was collected from a very shallow (less than 5 cm deep) geothermally heated pool (pH of around 9.0 and temperature range from about 30 °C at the edge to 70 °C in the centre at 5 cm into the sediment). At the water/sediment interface the sediment was grey; but below 3–5 mm into the sediment, it was black. The shallow pool was located a few metres away from a rusting iron container that collected hot spring water. The sample was kept at a laboratory in Athens, GA, USA, at 4 °C until it was used in 2001.

The isolation scheme included enrichments for thermophilic starch-degrading anaerobic bacteria. The pre-reduced anaerobic mineral bicarbonate-buffered medium for the enrichment and initial cultivation contained (per litre): 0.3 g KH2PO4, 0.66 g (NH4)2SO4, 0.29 g NaCl, 0.1 g MgSO4·7H2O, 0.03 g CaCl2·2H2O, 8.4 g NaHCO3, 0.2 g yeast extract (Difco), 2.0 g soluble potato starch, 0.24 g Na2S·9H2O, 0.2 g cysteine·HCl, H2O and 1 mg resazurin and was supplemented with 5 ml l⁻¹ of trace element and vitamin solutions (Freier et al., 1988), respectively. The pH25 °C (Wiegel, 1981) was adjusted to 8.5 by 5 M NaOH before autoclaving. Incubations at 60 °C yielded turbid cultures within two weeks. Subsequently, subcultures were inoculated with a variety of mono and disaccharides (21 total) and yeast extract (YE) alone. Microscopy revealed that spor-forming rods prevailed on carbohydrate-containing media, whereas in the YE-containing media, smaller and longer non-spor-forming rods dominated. Subsequent transfers onto the media with increased YE content (5 g l⁻¹), incubated at 60 °C, yielded cultures with...
increased numbers of long, non-spore-forming cells. The pure culture was isolated anaerobically by picking colonies from repeated series of serial dilutions in agar shake-roll tubes (Ljungdahl & Wiegel, 1986) using agar-solidified (2.2 %, w/v) pre-reduced medium with an increased concentration of YE (10 g l⁻¹) but without the addition of starch. Under anaerobic conditions, small (about 1 mm), round translucent colonies with even edges and flat surfaces developed after 2 days. Agar-embedded colonies were lens shaped. The final isolate, strain JW/VK-KS5Yᵀ, was chosen for further characterization. The purity of the isolate obtained was confirmed microscopically and by the uniformity of the colonies and was corroborated by 16S rRNA gene sequence analysis.

Initially after isolation, the cells of strain JW/VK-KS5Yᵀ were rod shaped, 0.35–0.45 µm in width and 3.0–8.0 µm in length. After several years of cultivation, the cells grew predominantly in long chains of cells or in filaments sometimes curled into helices (Fig. 1a). The strain was observed to grow in clumps of cells that could be broken easily by a vortex mixer. In the early exponential growth phase, Y-shaped (branch-like) cells were occasionally observed (Fig. 1b), a feature shared for the other members of the genus Anaerobranca (Engle et al., 1995). Recently Y-shaped cells have also been observed with other thermophilic bacteria (Engle et al., 1996; Kevbrin et al., 2005). Overgrown cultures (two days at 60 °C) reverted back to single cells (Fig. 1c). Under all observed growth conditions, motility was never observed and flagella were never seen in electron micrographs of negatively contrasted cells (Fig. 2). Cells of the novel strain stained Gram-negative. However, the KOH test was negative. Electron micrographs revealed a Gram type positive cell wall (data not shown). Although phylogenetically belonging to the Gram type positive bacteria (Firmicutes) (Wiegel, 1981), species of the genus Anaerobranca stain either Gram-positive, as does the type species A. horikoshii (Engle et al., 1995), or stain Gram-negative at all growth phases as observed for strain JW/VK-KS5Yᵀ, A. gottschalkii (Prowe & Antranikian, 2001) and A. californiensis (Gorlenko et al., 2004). No spores were observed using microscopy and in agreement with this observation, viability was lost after heat treatment at 80 °C for 10 min.

For characterization of the isolate, growth was followed by monitoring the increase in optical density (OD) at 600 nm in Hungate tubes with a spectrophotometer (UNICO 2100; United Products & Instruments) using the medium described above with 0.5 % YE (w/v). All tests were performed at least in duplicate. A shaking temperature gradient incubator (Scientific Industries) was used to determine the temperature range for growth by plotting doubling time versus incubation temperature. Growth at pH 25 °C 8.5 occurred within the temperature range of 34 to 64 °C, (no growth at 32 °C or below or at 66 °C or above) with an optimum at 54–60 °C. For the pH 25 °C range determination, the medium described above was used with the following modifications (per litre): 4.2 g NaHCO₃, 4.18 g MOPS, 5.14 g TABS and 10.0 g YE. The pH values were roughly adjusted at room temperature with 5 M NaOH anaerobically prior to sterilization. After autoclaving and before growth, the pH 25 °C of each point was measured repeatedly to ensure the correct pH 25 °C for plotting the growth data using the doubling time determined at early exponential growth. Testing for sodium, carbonate and chloride requirements was carried out in 50 mM TABS-buffered medium. As the TABS and other Good’s buffers set an initial pH 25 °C of around 5.0 when dissolved, 5 M KOH was used to adjust the pH to 8.9. The final potassium content was around 35 mM. The novel strain grew within a pH 25 °C range of 7.7–9.9 (no growth at or below pH 25 °C 7.5 or at or above pH 25 °C 10.2), with an optimum at pH 25 °C 8.5–9.0. This classified the novel isolate as a true alkali-thermophile (Kevbrin et al., 2004). Strain JW/VK-KS5Yᵀ was an
obligate anaerobe and did not grow either under air or in microaerophilic, i.e. in pink (oxidized resazurin), medium.

Tests for a requirement for sodium were carried out in a 50 mM TABS-buffered medium. Substitution of all added sodium salts with potassium salts yielded only faint growth on two subsequent transfers. Optimal growth was observed in the presence of 0.1 to 0.25 M sodium ions. Concentrations of 0.5 M sodium ions were inhibitory and concentrations above 0.6 M of supplemented sodium ions prevented growth.

The novel isolate required YE for growth and grew well on medium with YE (3–5 g l\(^{-1}\)) as the sole carbon and energy source, reaching a final OD of about 0.2. When tested on medium with only 0.5 g l\(^{-1}\) of YE, only soytone (3 g l\(^{-1}\)), but not peptone or tryptone (all Difco), supported growth in subsequent transfers. However, the final OD reached was only 0.1 under these conditions. As YE is a complex mix of uncertain composition, various brands of YE were compared with a view to enhancing the final OD of the cultures. Among the YE tested (Difco, Sigma, Oxoid, ICN, Quest, Serva), the best one was Bacto yeast extract from BD Company (Difco) and this was subsequently used throughout the study.

Fermentation products were analysed using a Stayer model HPLC (Aquilon) equipped with a refractometric detector and an Aminex HPX-87H column (Bio-Rad) (Paavilainen & Korpela, 1993) operated isocratically using 5 mM H\(_2\)SO\(_4\) as eluent at 0.6 ml min\(^{-1}\). Hydrogen was assayed by a LKHM-80 model GC (Chromatograph, Russia) equipped with a thermal conductivity detector and a molecular sieve 5A column and operated at room temperature. The following products were determined in cultures grown with 5 g l\(^{-1}\) YE as the sole carbon and energy source: acetate (3.1 mM) hydrogen (2.9 mM) formate (1.9 mM) and propionate (0.7 mM) (CO\(_2\) was not measured). Amounts were corrected for the minute quantities of formate and acetate (no propionate) found in blank medium. A distinct disaccharide peak was observed in the controls and at the beginning of the culture but it disappeared during growth. The Difco & BBL Manual (Zimbro & Power, 2003) does not mention disaccharides as a component of their media. GC separation of trimethylsilyl-treated YE on capillary columns identified the peak as representing trehalose. No other disaccharides were found. In a 5 g l\(^{-1}\) solution of YE, the trehalose content was close to 1 mM (0.342 g l\(^{-1}\)). When the other common disaccharides were tested (cellobiose, maltose and sucrose), only trehalose-grown cultures completely fermented the disaccharide and exhibited the equivalent amounts of fermentation products. Sucrose and maltose were utilized less completely. Cellobiose was not utilized at a measurable rate. When YE was substituted with peptone, which does not contain trehalose, only weak growth was observed. However when supplementing with trehalose, the resulting OD and profile of products were comparable with cultures grown with Difco YE (see Supplementary Table S1 in IJSEM Online). No growth at all was observed in media containing Casamino acids in the presence or absence of added trehalose. As Casamino acids are manufactured by strong acid hydrolysis, no peptides, tryptophan or other complex molecules are present in this product (Zimbro & Power, 2003), whereas peptone and YE are products of mild, enzymic hydrolysis and retained some complex compounds. The YE supplied by Difco had the highest content of trehalose, which could be the reason why it was the best YE product for the growth of strain JW/VK-KS5Y\(^{T}\). Soytone also contains some trehalose (0.4 mM in a 5 g l\(^{-1}\) solution of soytone), although less than that found in Difco YE, and thus it supported the growth of culture only to a lower OD. This suggests that YE serves as a source of trehalose and as a source of some unknown peptides. Complex food requirements have been demonstrated previously for the type species of the genus, A. horikoshii, but a specific compound was not identified (Engle et al., 1995).

Substrate utilization and electron acceptor reduction tests were performed at the optimal temperature of 60 °C and pH\(_{25}^{\circ}\) of around 8.8 by using the optimal carbonate-buffered medium with the following composition (per litre): 6.72 g NaHCO\(_3\), 2.12 g Na\(_2\)CO\(_3\), 5.0 g YE and vitamins, but reductants were omitted. Mono- and disaccharides were prepared as anaerobic stock solutions in distilled water and added to the autoclaved medium. Other substrates were autoclaved with the medium. The final concentration for all tested substrates was 3 g l\(^{-1}\). Co-utilization and increased growth compared with the control with yeast extract alone was observed with trehalose, sucrose, maltose, pyruvate, glucose, starch, pectin and polygalacturonate (the Sigma brand for two latter substrates contained 95 % main substance; Supplementary Table S1). The formation of nearly equal

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**Fig. 2.** Electron micrograph of cells of strain JW/VK-KS5YT. Negatively contrasted cells were stained by phosphotungstic acid. Bar, 1 μm.
quantities of formate (8.9 mM) and acetate (11.4 mM) and only low amounts of H₂ (2.7 mM) from 9.3 mM of utilized pyruvate (see Supplementary Table S1) indicated that pyruvate was mainly metabolized via pyruvate:formate lyase and not via the pyruvate:ferredoxin oxidoreductase commonly found in *Firmicutes*. In the latter case, acetate plus H₂ would dominate. Pyruvate:formate lyase is common for low G+C Gram-type positive bacteria (Karlin et al., 2004). Utilization of pyruvate has been shown previously for *A. californiensis* (Gorlenko et al., 2004), but the fermentation products were not identified. Strain JW/VK-KS5YT possessed hydrolytic enzymes for pectin and polygalacturonate but the presence or absence of specific enzymes [e.g. polygalacturonidase (exo), galaturonase (endo), esterase, specific pectinases] was not confirmed. The formation of 0.5 mM galacturionate, the monomeric unit of the polymer, as a product in both cases (which was not utilized when added as the carbon source) suggested the presence of a polygalacturonidase. Pectin and polygalacturonate hydrolytic enzyme activities were observed in this study for the first time for species of the genus *Anaerobranca*.

In media with low (0.05% w/v) YE concentration, with or without addition of the double concentration (see above media) of vitamins, strain JW/VK-KS5YT did not grow demonstrably (evaluated both by increase in OD and in quantitative comparison of the product profiles) in media supplemented with 0.3% (w/v), arabinose, xylose, ribose, xylitol, mannitol, sorbitol, inositol, dulcitol, glucose, fructose, galactose, mannose, rhamnose, sucrose, malose, trehalose, cellobiose, lactose, trehalose, raffinose, glycerol, 2,3-butanediol, erythritol, acetoin, salicin, pyruvate, fumarate, lactate, tartrate, malate, malonate, succinate, glycolate, gluconate, galacturonate, lactate, peptone, tryptone, Casamino acids, guanine, adenine, cytosine, thymine, uracil, pectin, starch, xylan, gelatin, casein, cellulose (filter paper) or Tween 80.

In the presence of 0.5% YE as electron donor, strain JW/VK-KS5YT reduced the following compounds (supplied at 10 mM concentrations): fumarate to succinate (9.3 mM), thiosulfate to a mix of sulfide (5.7 mM) and sulfite (3.7 mM), and elemental sulfur (10 g l⁻¹) to sulfide (8.3 mM) (Supplementary Table S1). Sulfide was assayed colorimetrically (methylene blue formation; Trüper & Schlegel, 1964). Thiosulfate and sulfite were quantified titrimetrically with iodine and formaldehyde as a blocking reagent (Gorlenko et al., 2004). Due to the reducing power of the essential YE at the elevated temperature, and/or the formation of iron reducing compounds from the yeast extract (such as sulfide from cystine and/or methionine), the dissimilatory reduction of Fe(III) citrate to Fe(II) could not be unequivocally demonstrated (no or only traces of magnetite formation) despite the fact that no Fe(III) was left after growth and uninoculated controls did not show either formation of black sediment or removal of Fe(III).

The reduction of thiosulfate to sulfide and sulfate has been reported previously for *A. californiensis* (Gorlenko et al., 2004). Thus this is the second report of thiosulfate reduction with sulfide and sulfate as products. When elemental sulfur is not found as a product of thiosulfate utilization, researchers usually look for sulfide alone. Thus, the above process might be underestimated in nature and could be more widespread than presently assumed. Notably, reduction of all acceptors was accompanied by the formation of lower levels of hydrogen and formate and by the appearance of trace but distinctive levels of organic isocids that were otherwise not observed with the above-mentioned fermentable co-substrates.

The addition of sulfate, nitrate, DMSO, acetone (10 mM of each) and sulfite (5 mM), did not enhance growth (final ODs were comparable with controls) and their corresponding reduction products were not observed. The addition of nitrite (5 mM) and trimethylamine N-oxide (10 mM) completely inhibited growth. Although biological reduction of selenite has previously been found for the three other recognized species of the genus *Anaerobranca* by Gorlenko et al. (2004), the reduction of selenite by strain JW/VK-KS5YT could not be established due to the spontaneous reduction of selenite to red elemental selenium at the required incubation conditions (60 °C, pH 25 °C 8.9, 5 g l⁻¹ YE).

The shortest observed doubling time was 28 min which was achieved using YE-supplemented medium (1%, w/v) plus 1.6 g l⁻¹ of sodium fumarate.

DNA isolation, amplification, sequencing and analysis of the 16S rRNA gene nucleotide sequence and DNA G+C content determination were carried out as previously described (Garnova et al., 2003). The genomic DNA G+C content was 32.5 mol% and was within the range of values observed for the other species of the genus (Table 1). The almost complete (1456 bp) sequence of the 16S rRNA gene of strain JW/VK-KS5YT was aligned and compared with sequences from related bacteria. The **FASTA** search engine at the EMBL website (Pearson & Lipman, 1988) was used for finding sequences that were close relatives to strain JW/VK-KS5YT. Retrieved sequences were aligned by using the **CLUSTAL_X** program (Thompson et al., 1997) and were manually edited. An unrooted phylogenetic tree was inferred applying the **TREECON** program (Van de Peer & De Wachter, 1994). The set of sequences was bootstrapped and the distance matrix was estimated for each tree according to the Jukes–Cantor formula. The resulting tree topology was calculated according to the neighbour-joining method. The inferred phylogenetic tree (Fig. 3) revealed that strain JW/VK-KS5YT clustered with representatives of the genus *Anaerobranca*. This genus currently has three recognized species, *A. horikoshii*, *A. gottschalkii* and *A. californiensis*. All of them are rod-shaped, non-spore-forming motile bacteria that are thermophilic, facultative or truly alkaliphilic and have a DNA G+C content of 30–34 mol%. The DNA G+C content of strain JW/VK-KS5YT was 32.5 mol%. The levels of 16S rRNA gene sequence
similarity between strain JW/VK-KS5YT and A. horikoshii JW/YL-138T, A. californiensis PAOHA-1T and A. gottschalkii LBS3T were 99.6 %, 98.5 % and 97.3 %, respectively. The closest neighbour to strain JW/VK-KS5YT was the type species of the genus, A. horikoshii. The DNA–DNA hybridization level between strain JW/VK-KS5YT and A. horikoshii DSM 9786T was determined as described by Garnova et al. (2003). The DNA–DNA relatedness value of 51 % clearly suggested that the strains represented two separate species.

Compared with other species of the genus Anaerobranca, strain JW/VK-KS5YT varied in several traits (Table 1). The main phenotypic differences between strain JW/VK-KS5YT and A. horikoshii were that strain JW/VK-KS5YT was non-motile, was a true alkaliphile (i.e. did not grow at pH \(25^\circ C \geq 7.0\)), stained Gram negative, grew on complex proteinaceous substrates that provided it with trehalose (YE and soytone or peptone together with trehalose) and that it formed nearly equimolar concentrations of acetate and formate from the carbon and energy source YE. On the basis of phenotypic, phylogenetic and DNA–DNA hybridization data, it is proposed that strain JW/VK-KS5YT represents the type strain of a novel species, Anaerobranca zavarzinii sp. nov.

<table>
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<td>0.26–0.31 × 2.4–5.0</td>
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<td>Fermentation products</td>
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<td>From glucose or starch: Acetate, ethanol (traces)</td>
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**Table 1.** Comparative features of recognized members of the genus Anaerobranca and strain JW/VK-KS5YT

Strains: 1, JW/VK-KS5YT; 2, A. horikoshii JW/YL-138T (Engle et al., 1995); 3, A. gottschalkii LBS3T (Prowe & Antranikian, 2001); 4, A. californiensis PAOHA-1T (Gorlenko et al., 2004). +, Positive; –, negative; NR, not reported. All strains grow on YE and are able to reduce thiosulfate and elemental sulfur (Gorlenko et al., 2004).

**Fig. 3.** Phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of strain JW/VK-KS5YT in the Anaerobranca cluster. Bootstrapping was based on 100 resampled datasets. Bar, 0.05 nucleotide substitutions per position.
Description of Anaerobranca zavarzinii sp. nov.

Anaerobranca zavarzinii (za.var.zi’ni.i. N.L. masc. gen. n. zavarzinii of Zavarzin, named after the Russian microbiologist George Zavarzin, in recognition of his seminal contributions to the knowledge of alkaliphilic microorganisms and their ecology).

Cells are rod-shaped, filamentous and infrequently branching in young cultures. Cells are 0.35–0.45 μm wide and 3.6–7.7 μm long. Cells have the tendency to grow in long chains. Division occurs by binary fission. Neither spores nor flagella are observed. Colonies are up to 1 mm in diameter, translucent and round with an even edge and a flat surface. The morphology of the cell wall is of the Gram-positive type, but the cells stain Gram-negative under all growth conditions. Growth temperature ranges from 34 to 64 °C, with an optimum of 54–60 °C (no growth at or below 32 °C or at or above 66 °C). The pH25°C range for growth is 7.7–9.9, with an optimum of pH25°C 8.5–9.0. No growth is observed at or below pH25°C 7.5 or at or above pH25°C 10.2. Growth occurs at total sodium concentrations over the range 20 to 540 mM, with a broad optimum at 120–240 mM. Obligately anaerobic chemo-organotroph. Pectin and polygalacturionate are hydrolysed. The best substrate for growth is 0.5% (w/v) YE which can be replaced by 0.5% (w/v) peptone plus >1 mM trehalose. In the presence of YE, trehalose, sucrose, maltose, pyruvate, starch and glucose are fermented and enhanced growth is seen compared with YE alone. Fermentation products on YE alone are acetate (3.1 mM), hydrogen (2.9 mM), formate (1.9 mM) and propionate (0.7 mM) (CO2 is assumed to be produced but the concentration has not been determined). Pyruvate is fermented to equimolar amounts of acetate and formate. Able to reduce fumarate to succinate, thiosulfate to a mixture of sulfate and sulfite and elemental sulfur is reduced to sulfide. Growth is enhanced by all co-substrates, fumarate and thiosulfate.

The type strain, JW/VK-KSSY7 (=VKM B-2436T=DSM 18970T), was isolated from a geothermally heated small and shallow water pool located at Pushino (Kamchatka, Russia). The DNA G+C content of the type strain is 32.5 mol%.

Acknowledgements

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References


