Desulfitibacter alkalitolerans gen. nov., sp. nov., an anaerobic, alkalitolerant, sulfite-reducing bacterium isolated from a district heating plant

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A novel alkalitolerant, anaerobic bacterium, designated strain sk.kt5^T, was isolated from a metal coupon retrieved from a corrosion-monitoring reactor of a Danish district heating plant (Skanderborg, Jutland). The cells of strain sk.kt5^T were motile, rod-shaped (0.4–0.6 × 2.5–9.6 µm), stained Gram-positive and formed endospores. Strain sk.kt5^T grew at pH 7.6–10.5 (with optimum growth at pH 8.0–9.5), at temperatures in the range 23–44 °C (with optimum growth at 35–37 °C), at NaCl concentrations in the range 0–5% (w/v) (with optimum growth at 0–0.5%) and required yeast extract for growth. Only a limited number of substrates were utilized as electron donors, including betaine, formate, lactate, methanol, choline and pyruvate. Elemental sulfur, sulfite, thiosulfate, nitrate and nitrite, but not sulfate or Fe(III) citrate, were used as electron acceptors. The G + C content of the DNA was 41.6 mol%. Phylogenetic analyses of the sequence data for the *dsrAB* genes [encoding the major subunits of dissimilatory (bi)sulfite reductase] and the 16S rRNA gene placed strain sk.kt5^T within a novel lineage in the class *Clostridia* of the phylum *Firmicutes*. Taken together, the physiological and genotypic data suggest that strain sk.kt5^T represents a novel species within a novel genus, for which the name *Desulfitibacter alkalitolerans* gen. nov., sp. nov. is proposed. The type strain of *Desulfitibacter alkalitolerans* is sk.kt5^T (=JCM 12761^T=DSM 16504^T).

The pipe networks of district heating systems constitute a unique aqueous environment, characterized by anoxia, low nutrient availability, high pH (9.5–10.0) and relatively high temperatures (≥40 °C). These extreme conditions serve to minimize chemical corrosion and limit bacterial growth. However, oligotrophic conditions may not be sufficient to prevent biofilm formation in a flow system, since nutrients are constantly being replenished and tend to concentrate on submerged surfaces, thus promoting attached bacterial growth. Such biofilm formation can locally alter the surface chemistry, which in turn can result in (bio)corrosion of the surface (Hamilton, 1995; Little *et al.*, 1990). Sulfate-reducing bacteria are generally considered as the major causative agent of biocorrosion because of their ability to produce corrosive hydrogen sulfide and/or to consume molecular hydrogen (Lee *et al.*, 1995; Hamilton, 1995). Biocorrosion is of particular importance in high-sulfate marine technical systems, especially those in the offshore oil industry, and can cause substantial economic losses (Marshall, 1992). However, biocorrosion can also occur in low-sulfate freshwater systems such as district heating

networks, as was highlighted by recent surveys showing that several Danish district heating systems occasionally suffer from biocorrosion (Kjellerup *et al.*, 2003, 2005). Attempts by those authors to link the occurrence of biocorrosion specifically to the presence of sulfate-reducing bacteria proved unsuccessful, suggesting that other types of micro-organisms might also be involved in biocorrosion processes.

Here, we describe a novel alkalitolerant, sulfide-producing, anaerobic bacterium, strain sk.kt5^T, isolated from a biofilm sample from a Danish district heating system that had previously suffered from failures due to biocorrosion.

Strain sk.kt5^T was enriched from biofilms that developed on mild steel coupons during a 4 month incubation period in a corrosion-monitoring reactor connected to the return line (bulk water pH 9.5–10.0, 40 °C) of Skanderborg District Heating Plant in Denmark. The water-quality parameters of this system have been reported previously (Abildgaard *et al.*, 2006). Enrichment cultures were initiated by inoculating biofilm material aseptically scraped off metal coupons and suspended in approximately 1 ml filter-sterilized (0.2 µm pore size) district heating water into a modified version of Postgate's medium B (Postgate, 1984) containing the following (l⁻¹ MilliQ water): 0.5 g KH₂PO₄, 0.25 g NH₄Cl, 1.26 g CaSO₄·2H₂O, 1.0 g MgSO₄·7H₂O, 0.1 g

The GenBank/EMBL/DDBJ accession numbers for the *dsrAB* and 16S rRNA gene sequences of strain sk.kt5^T are AY538171 and AY751285, respectively.

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g yeast extract, 1.0 ml trace element mixture (Widdel & Bak, 1992), 1.0 ml selenite/tungstate mixture (Widdel & Bak, 1992) and 50 μl resazurin solution (2 %, w/v). After sterilization of the medium, the following solutions were added aseptically from sterile anoxic stocks (1^{-1} medium): 1.0 ml vitamin mixture [modified after Widdel & Bak (1992) by including 30 mg folic acid 1^{-1}], 1.0 ml vitamin B_{12} solution and 1.0 ml thiamine solution (Widdel & Bak, 1992). The following substrates were each added to a final concentration of 5 mM: propionate, acetate, lactate and ethanol. Before use, the pH of the medium was adjusted to 9.0 using sterile, anoxic NaOH (4 M) and the medium was finally reduced by adding a 2 % (w/v) sodium dithionite solution (3 ml 1^{-1}). Enrichment cultures were incubated at 40 °C in the dark. Strain sk.kt5^T was isolated from one of the enrichment cultures by repeated (3 ×) application of the roll-tube technique (Hungate, 1976), using the same medium as described above but further supplemented with 10 mM thiosulfate and washed agar (2 %, w/v). The purity of the isolated culture was examined by phase-contrast microscopy after growth (at pH 7.0, 8.0 and 9.0) in the above-mentioned medium supplemented with 20 mM glucose, 20 mM lactate and 10 mM thiosulfate.

Routine cultivation and all growth tests were performed in 16 × 125 mm Hungate anaerobic culture tubes (Bellco Glass) containing 10 ml basal medium buffered with CAPSO (3-cyclohexylamino-2-hydroxy-1-propanesulfonic acid, $\text{p}K_a$ 9.6) prepared as described previously (Abildgaard *et al.*, 2006). Unless noted otherwise, the pH of the basal medium was adjusted to 9.0 (measured at 40 °C), the medium was supplemented with betaine and thiosulfate (20 mM each) and all incubations were carried out in duplicate at 40 °C in the dark. Cell growth was quantified by measuring optical density at 600 nm (OD_{600}). Because of the formation of inorganic precipitates in the basal medium at pH values greater than 9.0, OD_{600} measurements were sometimes supplemented with measurements of sulfide production (performed using the method of Cline, 1969) and with total counts of SYBR Gold-stained cells (performed according to Mogensen *et al.*, 2005). Transmission electron microscopy was performed as described previously (Mogensen *et al.*, 2005). The Gram-staining reaction was determined by using a standard procedure. The pH range for growth was determined at 11 different pH values ranging from 6.3 to 10.5, obtained by titrating the medium with sterile, anoxic 2 M HCl or NaOH solutions. The pH did not remain stable when titrated to values above 10.5, so growth of the strain was not evaluated at higher pH. The temperature range for growth was determined using a temperature-gradient block (Elsgaard *et al.*, 1994) at 21 different temperatures ranging from 0.6 to 50.2 °C. The effect of NaCl on growth was determined at 0.1, 0.5, 1.0, 2.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 10, 15 and 20 % (w/v). The ability of strain sk.kt5^T to utilize different electron donors was tested in separate incubations using both thiosulfate (20 mM) and sulfite (10 mM) as electron acceptors. The fermentative abilities of strain sk.kt5^T were tested in

medium devoid of external electron acceptors. The utilization of different electron acceptors was tested using betaine (20 mM) and yeast extract (0.5 g 1^{-1}) as electron donors. Cultures grown in medium with 20 mM betaine and 0.5 g 1^{-1} yeast extract without any external electron acceptors were used as inocula (5 %, v/v) for the latter experiments. The reduction of Fe(III) citrate (20 mM) was examined in unreduced medium by measuring the Fe(II) concentration by means of the ferrozine method (Lovley & Phillips, 1986). Notably, strain sk.kt5^T was capable of initiating growth in unreduced medium when the inoculum was from betaine-grown cultures. The reduction of nitrate (20 mM) and nitrite (2 mM) was tested (five replicate cultures each) by measuring the OD_{600} and by monitoring the concentrations of nitrate and nitrite using HPLC (Kjeldsen *et al.*, 2004), as well as by assessing the ammonium concentration by using a colorimetric method (Bower & Holm-Hansen, 1980). Plating on agar plates (1.5 %, w/v) consisting of basal medium (pH 9.0) was used to test for aerobic growth.

The cells of strain sk.kt5^T were motile straight rods varying in size from 0.4 to 0.6 μm in diameter and from 2.5 to 9.6 μm in length (Fig. 1). Strain sk.kt5^T stained Gram-positive and formed round terminal endospores (Fig. 1). The temperature range for growth was 23–44 °C, with an optimum at 35–37 °C. After 120 days incubation, growth was detected at temperatures down to 14.5 °C. No growth

Fig. 1. Transmission electron micrographs of uranyl acetate-stained cells of strain sk.kt5^T. (a) Dividing cells. (b) Cell with polar flagellum. (c) Cell with terminal endospore. Bars, 1.0 μm (a, c) and 2.0 μm (b).

was detected at 48 °C or at ≤ 11 °C. Cultures of strain sk.kt5^T survived pasteurization for 4 h at 60 °C. The pH range for growth was pH 7.6 to approximately pH 10.5 (strain sk.kt5^T exhibited only weak growth at this pH; higher values were not tested, as mentioned above); optimum growth occurred at pH 8.0–9.5. Growth was not observed at or below pH 7.5. Strain sk.kt5^T grew at NaCl concentrations ranging from 0 to 5 % (w/v), the optimum being observed at 0–0.5 % (w/v) NaCl.

Yeast extract was required for growth and could not be replaced by a vitamin mixture. Strain sk.kt5^T grew on yeast extract (0.5 g l⁻¹) as the sole substrate, both fermentatively and with thiosulfate or sulfite present as the electron acceptor. Under the latter conditions, the observed increases in OD₆₀₀ values during incubation ranged from 0.05 to 0.07. Therefore, in growth tests, only those compounds yielding OD₆₀₀ increments of at least 0.09 were scored as growth substrates. In the presence of thiosulfate (20 mM), strain sk.kt5^T grew on betaine (30 mM), formate (50 mM), lactate (30 mM), methanol (50 mM) and pyruvate (40 mM), with OD₆₀₀ values ranging from 0.09 to 0.17. The sulfide concentrations produced with these electron donors varied between 2.0 and 6.8 mM. In the presence of sulfite (10 mM), the strain also grew on choline (30 mM) (but not on formate at 50 mM), with OD₆₀₀ values ranging from 0.12 to 0.23, and produced sulfide concentrations varying between 5.0 and 10.0 mM. In the presence of thiosulfate or sulfite, the strain did not grow on H₂/CO₂, acetate (20 mM), acetone (20 mM), D(–)-arabinose (20 mM), benzoate (30 mM), 2-butanol (40 mM), butyrate (40 mM), Casamino acids (30 mM), choline (30 mM), ethanol (50 mM), D(–)-fructose (30 mM), fumarate (20 mM), D(+)–galactose (20 mM), D(+)–glucose (20 mM), glycerol (30 mM), glycine (40 mM), DL-malate (30 mM), D(+)–mannose (30 mM), 1-pentanol (30 mM), 2-propanol (50 mM), propionate (40 mM), L(+)–rhamnose (30 mM), succinate (40 mM), sucrose (20 mM) or D(+)–xylose (30 mM). Besides fermenting yeast extract, strain sk.kt5^T was able to grow fermentatively (at low growth rates) on betaine (20 mM). With betaine as the electron donor, strain sk.kt5^T reduced elemental sulfur, sulfite and thiosulfate to sulfide. Both nitrate and nitrite were reduced, but nitrite inhibited growth at concentrations of 2 mM or more. Apparently neither N₂ (as evident from the absence of gas production) nor ammonium was an end-product of nitrate or of nitrite reduction. Strain sk.kt5^T did not reduce sulfate or Fe(III) citrate. The strain did not grow under oxic conditions.

Genomic DNA of strain sk.kt5^T was extracted and the *dsrAB* [encoding the alpha and beta subunits of dissimilatory (bi)sulfite reductase] and 16S rRNA gene sequences were retrieved as described previously (Abildgaard *et al.*, 2006). A custom-designed internal *dsrAB* sequencing primer (5′-GATGCATGCACTGTATTAACAAA-3′, annealing to *Desulfovibrio vulgaris dsrA* positions 728–751) was used to complete the *dsrAB* sequence of strain sk.kt5^T. The 16S rRNA gene sequence of strain sk.kt5^T was aligned according

to the ssu_jan04_corr_opt.arb ARB database (available at <http://www.arb-home.de>). Phylogenetic trees (based on datasets of sequences >1350 nt) were constructed using the neighbour-joining (with Jukes–Cantor distance correction), maximum-parsimony and maximum-likelihood algorithms of the ARB package (Ludwig *et al.*, 2004). A 50 % conservation filter calculated for the taxa shown in Fig. 2(a) was applied to select sequence positions (1277 nt) for the analyses. The phylogenetic analysis of the *dsrAB* sequence of strain sk.kt5^T was carried out as described previously (Abildgaard *et al.*, 2006) and included 457 unambiguously aligned amino acid sequence positions. Bootstrap analyses were performed and phylogenetic consensus trees were constructed as described previously (Abildgaard *et al.*, 2006).

Initial phylogenetic analysis of the 16S rRNA gene sequence of strain sk.kt5^T, performed using BLAST searches, revealed that the novel strain has no close relatives among recognized bacterial species, the highest level of similarity (89 %) being shared with members of the Gram-positive genus *Moorella* (*Moorella thermoacetica* DSM 521^T and *Moorella thermoautotrophica* DSM 1974^T). However, >97 % similarity was shared with a number of environmentally derived 16S rRNA gene sequence clones representing uncultivated bacteria originating from alkaline water samples from a South African gold mine (Baker *et al.*, 2003; GenBank accession numbers AF486695, AY540791, AY540795, AY540811, AY540821 and AY540824). Phylogenetic treeing consistently placed strain sk.kt5^T within the class *Clostridia* of the phylum *Firmicutes* as a novel sister lineage to the genus *Moorella* (Fig. 2a). Calculation of pairwise similarities from full-length alignment of all taxa included in the tree shown in Fig. 2(a), using the *Firmicutes* filter of the ssu_jan04_corr_opt.arb ARB database or a filter based on the Lane mask (which excludes all hypervariable sequence positions of the 16S rRNA gene; Lane, 1991), identified the two above-mentioned *Moorella* species and *Moorella glycerini* DSM 11254^T as the closest relatives of strain sk.kt5^T (91.5–91.8 % similarity). Similarity values that were 0.5–1.5 % lower were shared with several species belonging to different genera, including certain members of the sulfate-reducing genus *Desulfotomaculum*. Even lower levels of similarity (89.1–89.2 %) were shared with *Moorella mulderi* DSM 14980^T, which, together with the three above-mentioned species, constitute the only members of the genus *Moorella* that have validly published names. Like strain sk.kt5^T, the latter species are all anaerobic, Gram-positive, spore-forming rods capable of using thiosulfate as an electron acceptor (Fontaine *et al.*, 1942; Wiegel *et al.*, 1981; Ljungdahl, 1986; Slobodkin *et al.*, 1997; Balk *et al.*, 2003). However, apart from the above-mentioned similarities, a number of phenotypic and genotypic properties differentiate strain sk.kt5^T from this genus. Most notably, the genomic G+C content of strain sk.kt5^T (41.6 mol%; determined by HPLC analysis at the Identification Service of Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) differs by more

Fig. 2. Unrooted consensus trees showing the phylogenetic affiliation of the 16S rRNA gene (a) and DsrAB amino acid (b) sequences of strain sk.kt5^T. Most of the taxa shown in the 16S rRNA gene sequence-based tree are members of the class *Clostridia*. Multifurcations connect branches that were not resolved unambiguously by distance-matrix, maximum-parsimony and maximum-likelihood treeing methods. Numerals in square brackets indicate the numbers of genera and/or named species constituting the respective grouping. A number of outgroup sequences used to root the tree were subsequently removed. The node connecting the *Moorella* species and strain sk.kt5^T lineages in the 16S rRNA gene sequence-based tree received 74 and 61 % distance-matrix-based and maximum-parsimony-based bootstrap support, respectively. In the DsrAB-based tree, the node connecting the *Pelotomaculum* sp. MGP and strain sk.kt5^T lineages received 90 and 73 % distance-matrix-based and maximum-parsimony-based bootstrap support, respectively. Symbols: *, also including *Pelospira glutarica* DSM 6652^T (GenBank accession no. AJ251214) and *Syntrophospora bryantii* DSM 3014A^T (M26491); †, also including *Sporotomaculum hydroxybenzoicum* DSM 5475^T (Y14845); ‡, a partial *dsrAB* sequence of *Ammonifex degensii* DSM 10501^T was obtained through the NCBI homepage (<http://www.ncbi.nlm.nih.gov>) from the trace archive of whole genome shotgun reads from this organism (TI number 662586343). Bars, 10 % sequence divergence inferred from distance-matrix analyses.

than 12 mol% from the G + C contents of the four *Moorella* species, which suggests that strain sk.kt5^T should be placed outside this genus (Vandamme *et al.*, 1996). The genus

Moorella belongs to the family *Thermoanaerobiaceae*; however, as can be seen from the consensus tree shown in Fig. 2(a), the phylogenetic analyses used failed to resolve

the phylogenetic relationships among the families of the class *Clostridia*. Thus, strain sk.kt5^T cannot be confidently assigned to a family.

According to phylogenetic analyses based on DsrAB amino acid sequences, strain sk.kt5^T constitutes a novel lineage related to that of the syntrophic Gram-positive bacterium *Pelotomaculum* sp. MGP (Fig. 2b), which, like strain sk.kt5^T, is unable to reduce sulfate (Imachi *et al.*, 2006). Unfortunately, *dsrAB* sequence information is available only for a single *Moorella* species, namely *M. thermoacetica*. As can be seen from Fig. 2(b), this sequence clusters together with the xenologous *dsrAB* sequences of certain members of the class *Clostridia*, which probably acquired their *dsrAB* sequences by lateral gene transfer from a deltaproteobacterial donor related to *Desulfobacterium anilini* DSM 4660^T (Zverlov *et al.*, 2005). In contrast, strain sk.kt5^T probably carries an orthologous *dsrAB* gene, as shown by the congruence between its *dsrAB* and 16S rRNA gene sequence-derived phylogenies (Fig. 2a, b).

The genotypic and phenotypic analyses clearly differentiate sk.kt5^T from its 16S rRNA gene sequence-based phylogenetically closest relatives. Thus, we propose that strain sk.kt5^T should be considered as representing a novel species within a novel genus, for which the name *Desulfitibacter alkalitolerans* gen. nov., sp. nov. is proposed. *Desulfitibacter alkalitolerans* was isolated from biofilms grown on mild steel coupons in a reactor connected to a district heating plant exhibiting problems with biofilm formation and corrosion. Strain sk.kt5^T is capable of sulfide production through the reduction of elemental sulfur, thiosulfate or sulfite. The latter compounds could be formed transiently in the district heating system as a result of the chemical oxidation of biologically produced sulfide when oxygen is temporary introduced during maintenance/repair of the pipe system. As described in a separate publication (Abildgaard *et al.*, 2006), a novel sulfate-reducing bacterium, *Desulfovibrio alkalitolerans* DSM 16529^T, was recently isolated from a biofilm sample of the same district heating plant.

Description of the genus *Desulfitibacter* gen. nov.

Desulfitibacter (De.sul.fi.ti.bac'ter. L. pref. *de* from, off, away; N.L. n. *sulfis* -itis sulfite; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Desulfitibacter* rod-shaped bacterium that reduces sulfite).

Motile, Gram-positive rods. Form terminal endospores. Anaerobic, growing chemoheterotrophically by fermentation or by reduction of sulfite or thiosulfate. The type species is *Desulfitibacter alkalitolerans*.

Description of *Desulfitibacter alkalitolerans* sp. nov.

Desulfitibacter alkalitolerans (N.L. n. *alkali* alkali; L. pres. part. *tolerans* tolerating; N.L. part. adj. *alkalitolerans* alkali-tolerating).

Displays the following properties in addition to those given in the genus description. Cells are 0.4–0.6 × 2.5–9.6 µm in size, alkalitolerant and mesophilic. The temperature range for growth is 23–44 °C, with an optimum at 35–37 °C. Extended incubation (130 days) facilitates growth at temperatures down to 14.5 °C. The pH range for growth at 40 °C is 7.6–10.5, with an optimum at pH 8.0–9.5. NaCl concentrations up to 5 % (w/v) are tolerated, but the optimal concentration for growth is 0–0.5 % (w/v). Yeast extract is required for growth. The following substrates are utilized as carbon and energy sources in the presence of thiosulfate: betaine, formate, lactate, methanol and pyruvate. In the presence of sulfite, choline is also utilized, but formate is not. The following substrates do not support growth: H₂/CO₂, acetate, acetone, D(–)-arabinose, benzoate, 2-butanol, butyrate, Casamino acids, choline chloride, ethanol, D(–)-fructose, fumarate, D(+)–galactose, D(+)–glucose, glycerol, glycine, DL-malate, D(+)–mannose, 1-pentanol, 2-propanol, propionate, L(+)–rhamnose, succinate, sucrose and D(+)–xylose. Elemental sulfur, sulfite, thiosulfate, nitrate and nitrite (at low concentrations) are utilized as electron acceptors. Sulfate and Fe(III) citrate are not reduced. The DNA G + C content is 41.6 mol%.

The type strain, sk.kt5^T (=DSM 16504^T=JCM 12761^T), was isolated from a biofilm growing in a high-pH district heating system in Denmark.

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