Isolation of thermophilic Desulfotomaculum strains with methanol and sulfite from solfataric mud pools, and characterization of Desulfotomaculum solfataricum sp. nov.

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Four strains of thermophilic, endospore-forming, sulfate-reducing bacteria were enriched and isolated from hot solfataric fields in the Krafla area of north-east Iceland, using methanol and sulfite as substrates. Morphologically, these strains resembled thermophilic Desulfotomaculum species. The strains grew with alcohols, including methanol, with glucose and fructose as electron donors, and with sulfate, sulfite or thiosulfate as electron acceptors. For all four strains, the optimum temperature and pH for growth were 60 °C and pH 7–3, respectively; no added NaCl was required. Phylogenetic analysis based on partial 16S rRNA gene sequence comparisons showed high levels of similarity of the novel strains (> 92 %) with Desulfotomaculum kuznetsovii and Desulfotomaculum luciae. However, DNA–DNA hybridization studies with D. kuznetsovii revealed that the four strains belonged to one novel species. A representative of this group of isolates, strain V21T, is proposed as the type strain of a novel species of the spore-forming, sulfate-reducing genus Desulfotomaculum, namely Desulfotomaculum solfataricum (type strain V21T = DSM 14956T = CIP 107984T).

INTRODUCTION

Thermophilic, sulfate-reducing bacteria are found in a wide range of environments including hot springs/geothermal groundwater (Zeikus et al., 1983; Daumas et al., 1988; Nazina et al., 1988; Love et al., 1993; Henry et al., 1994; Liu et al., 1997), fresh water (Elsgaard et al., 1994; Kuever et al., 1999), cold marine sediments (Isaksen et al., 1994), oilfields (Rosnes et al., 1991; Rees et al., 1995; Beeder et al., 1995; Tardy-Jacquenod et al., 1996; Nilsen et al., 1996), compost/manure (Fardeau et al., 1995; Pikuta et al., 2000) and anaerobic bioreactors (Min & Zinder, 1990; Tasaki et al., 1991; Weijma, 2000; Plugge et al., 2002). Most of these thermophiles belong to a phylogenetically coherent cluster of Gram-positive, spore-forming Desulfotomaculum species (Stackebrandt et al., 1997). Gram-negative, thermophilic, sulfate-reducing bacteria are members of the genera Thermodesulfbacterium, Thermodesulfovibrio, Thermodesulforhabdus or Desulfacinum (Henry et al., 1994; Rees et al., 1995; Rozanova et al., 2001; Sievert & Kuever, 2000; Sonne-Hansen & Ahring, 1999; Zeikus et al., 1983; Beeder et al., 1995). These Gram-negative sulfate-reducers are all characterized by a narrow substrate range in comparison with the thermophilic Desulfotomaculum species. Most of the thermophilic sulfate-reducers also use sulfite and thiosulfate as electron acceptors. Sulfate reduction is energetically less favourable than sulfite reduction. Sulfate has to be activated first at the expense of ATP to adenosine-5′-phosphosulfate by ATP-sulfurylase; this is followed by adenosine-5′-phosphosulfate reduction to sulfite and AMP (Widdel & Hansen, 1992). Thiosulfate as electron acceptor is energetically also more favourable than sulfate, and in freshwater sediments thiosulfate is used preferentially (Jørgensen & Bak, 1991). The aim of our work is to identify...
and characterize bacterial strains that may find application in a biological process for the thermophilic desulfurization of off-gases. For this process, thermophilic, methanol-utilizing, sulfite-reducing strains were considered essential. In nature, mesophilic sulfite-reducing bacteria, i.e. Desulfococcus species, are found which cannot reduce sulfate (Utkin et al., 1994; Christiansen & Ahring, 1996; Gerrits et al., 1996, 1999; Bouchard et al., 1996; Sanford et al., 1996).

An appropriate sampling site for the isolation of thermophilic sulfite-reducers is the Krafla region in north-east Iceland, a relatively young, geothermically active area with recent volcanic activity. On the slopes of these young craters and in the lower region’s solfataric mud pools, large differences in temperature (40–110°C) and acidity (pH 2.5–8.0) were found. Enrichments from sediments of these pools led to the isolation of novel methanol-utilizing, sulfite- and sulfite-reducing bacteria. In this report, we describe the isolation and characterization of strain V21T.

**METHODS**

**Sources of cultures.** Desulfotomaculum kuznetsovii DSM 6115T was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Desulfotomaculum luciae was kindly provided by David Boone (Oregon Graduate Institute of Science and Technology, Portland, OR, USA). Strain TPOS was isolated at the Laboratory of Microbiology in Wageningen from anaerobic methanogenic granular sludge with propionate and sulfate as substrates (A. J. M. Stams, unpublished data). Strain WWI was enriched and isolated from a thermophilic methanol-fed, sulfite-reducing, laboratory-scale reactor and was kindly provided by Jan Weijma (J. Weijma, M. Balk & A. J. M. Stams, unpublished data). Desulfotomaculum thermobenzoicum subsp. thermosyntrophicum DSM 14053T was present in our culture collection.

**Source of inocula.** Sediment samples were taken from hot (45–110 °C) solfataric fields in the Krafla region of north-east Iceland. Around 10 samples of both the Naumaskjard and Viti solfataras were collected from the blackened layers. Samples were kept under an anaerobic atmosphere (N2/CO2, 80:20), transported at room temperature, and used for enrichments within 7 days of sampling.

**Media and cultivation.** A bicarbonate-buffered medium was used for growth, enrichment, and isolation experiments. The basal medium contained the following (g l−1): NaHCO3 (4) (separately sterilized), Na2SO4 (2.8), MgCl2·6H2O (1.2), KCl (0.5), NH4Cl (0.3), KH2PO4 (0.2), CaCl2 (0.15) and Na2S·7H2O (0.3) (separately sterilized). The following additions were made from anoxic stock solutions and per litre of medium: 0.5 ml vitamin solution according to Stams et al. (1983): 0.1 μM Na2SeO3, 0.1 μM Na2WO4 and 1 ml trace-element solution SL 6, according to Pfennig & Lippert (1966). In standard growth experiments, yeast extract (1 g l−1), Difco) and NaCl (7 g l−1) were added to the culture medium.

**Enrichment and isolation.** Enrichments were carried out in batch and continuous cultures. Incubations in batch experiments were done at 60 °C at neutral pH in 120 ml bottles filled with 50 ml basal medium without sulfate, then supplemented with 10 mM methanol as electron donor, 10 mM sulfate as electron acceptor and 5 mM hydrogen sulfide to select for sulfide-tolerant organisms. Yeast extract was omitted. The headspace consisted of N2/CO2 (80:20). Under anaerobic conditions, approximately 5 ml sediment was added to the culture bottles. After growth was observed (4–5 weeks), sulfide production was analysed and positive cultures were transferred to fresh medium. Slow-growing organisms were enriched in a continuous culture vessel with glass and Tellon parts present. The influent contained a standard medium with 10 mM methanol as the growth-limiting substrate, 10 mM sulfate instead of sulfate, and 5 mM sulfide. The dilution rate was 0.005 h−1, the temperature was 60 °C, the pH was maintained at 7.3 by titration with HCl, and the stirring speed was 100 rpm. At constant culture density, the culture was used for isolation. Strains were obtained in pure culture by using agar shake dilution tubes. Black colonies from the highest dilutions with growth were used for three successive transfers in agar tubes and checked for purity.

**pH, temperature and NaCl concentration optima.** The effect of pH on growth was determined at 60 °C. The pH of the basal medium was adjusted to defined values (pH range 6–8.5) with sterile stock solutions of NaOH or HCl. The temperature range (37–75 °C) for growth was determined in basal medium at pH 7.5. The requirement for NaCl was determined in basal medium containing a concentration range of 0–5 % (w/v) NaCl.

**Electron-donor and electron-acceptor utilization.** The ability of the strains to utilize substrates was tested in basal medium supplemented with autoclaved or filter-sterilized substrates. Concentrations ranged from 5 to 20 mM and cultures were incubated for 2 weeks. The utilization of various electron acceptors was studied in basal medium containing lactate (20 mM) as electron donor. Electron acceptors were added from sterile stock solutions to a concentration of 10 mM.

**Analytical procedures.** Optical density was measured at a wavelength of 660 nm in a Starcoll colorimeter (R&D Mechatronics). Methanol, methane and fatty acids were analysed by GC as described previously (Heijthuijsen & Hansen, 1989). Sulfide was determined colorimetrically using the methylene blue method of Tripler & Schlegel (1964). Bacterial growth was determined by measuring the increase in OD660 the methanol consumption and the sulfide production.

**Phospholipid fatty acid analysis.** Bacterial cultures of strain V21T and D. kuznetsovii, grown on methanol and sulfate, and D. thermobenzoicum subsp. thermosyntrophicum, grown on pyruvate and sulfate, were harvested by centrifugation (20 000 g, 20 min, 4 °C) and pellets were directly extracted using a modified Bligh–Dyer extraction. The total lipid extract was fractionated on silic acid, and mild alkaline transmethylation was used to yield fatty acid methyl esters from the phospholipid fraction. Concentrations of individual phospholipid fatty acids as fatty acid methyl esters were determined by capillary GC, coupled with a flame ionization detector. Identification of phospholipid fatty acids was based on comparison of retention-time data with known standards (see Boschker et al., 1999, for further details).

**Phylogenetic analysis.**

**Partial 16S rRNA gene sequence analysis of the four isolates.** For the phenotypic characterization of isolates V20, V21T, V28 and V29 and strain TPOS, chromosomal DNA was isolated from a liquid culture as described previously (Van der Maarel et al., 1996). The 16S rRNA gene was selectively amplified by PCR, using oligonucleotide primers complementary to conserved regions of the bacterial 16S rRNA gene. The following primer pair was used: 5′-ACCTAATAGCCTA-TATAACGAGTTTTGATCTCCGTC-AG-3′ (positions 8–27, Escherichia coli numbering) and 5′-ATTGTAAG- AACCGCCGACGTTTACCTGGTCTCATT-3′ (positions 1492–1510, E. coli numbering). The PCR amplification products were sequenced with an Applied Biosystems 373A DNA sequencer using the Taq DyeDeoxy terminator cycle sequencing method and custom primers based on conserved regions.

**Full 16S rRNA gene sequence analysis of strain V21T.** Extraction of genomic DNA and PCR-mediated amplification of the
16S rRNA gene were carried out as described previously (Rainey et al., 1996). Purified PCR products were cloned using the pCR-Script SK+ cloning kit from Stratagene. Genomic DNA was extracted from positive clones. PCR-mediated amplification of the 16S rDNA and purification of the PCR product were carried out as described previously (Rainey et al., 1996). Purified PCR products were sequenced using the ABI PRISM DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) as directed by the manufacturer’s protocol. Sequence reactions were electrophoresed using an Applied Biosystems 373A DNA sequencer. Approximately 95% of the 16S rDNA gene sequence of strain V21T was determined.

Sequence alignment and construction of a phylogenetic tree. The assembled DNA sequences were aligned with 16S rDNA gene sequences of closely related strains found in the GenBank database using CLUSTAL W. A 16S-rDNA-based phylogenetic tree was constructed from a distance matrix based on the neighbour-joining method (Saitou & Nei, 1987), as implemented in the program TREECON (Van de Peer & De Wachter, 1995). A manual correction method was applied and tree topology was re-examined by using bootstrap analysis (100 replications).

DNA–DNA hybridizations. DNA was isolated and purified according to Marmur (1961). The DNA base content was determined by the thermal denaturation method (Owen et al., 1961) and DNA homology was determined by De Ley’s optical reassociation method (De Ley et al., 1970).

RESULTS AND DISCUSSION

Isolation of pure cultures

Two sites in the Krafla region were sampled: Naumaskjárd and Viti. Naumaskjárd is a relatively old solfataric field, although no cyanobacterial colonization was visible around the site. Samples were taken from nine solfatares ranging in temperature from 40 to 100°C. The Viti site was on the slope of a young volcano, and elemental sulfur was abundant around this site. Samples were taken from 11 solfatares with temperatures ranging from 50 to 90°C and pH values ranging from 2.5 to 5.5. Media with methanol and sulfite were inoculated with these sediments and incubated at 60°C. After incubation for 4 weeks, sulfide was produced in three out of 20 enrichment cultures; these positive cultures all originated from inocula from the Viti sediments. No methane or acetate was produced in any of the cultures. None of the cultures containing NaCl were able to grow at any temperature. Therefore, NaCl was omitted from the media. Yeast extract stimulated growth and a vitamin supplement was required for growth.

Morphological and physiological characteristics

Cells of all four isolates were non-motile, straight rods (3.5–5 × 1.5 μm) that were often observed in pairs or longer chains (Fig. I; supplementary data, http://ijs.sgmjournals.org). All four strains formed spores, which were spherical and central or subterminal, and sporulation caused swelling of the cells to a lemon-shaped appearance. Spores were not extremely resistant to heat sterilization, as was found for some thermophilic Desulfotomaculum strains (Goorissen, 2002). Their decimal reduction value at 120°C was below 3 min. The Gram stain result was negative, as is observed often for Desulfotomaculum strains (Sleytr et al., 1969; Rosnes et al., 1991; Liu et al., 1997), but electron microscopic analysis revealed a typical Gram-positive cell wall architecture (results not shown). All four strains grew at temperatures between 48 and 65°C, the optimum growth temperature being 60°C. No growth was observed outside this temperature range. Growth occurred at initial pH values between 6.4 and 7.9. The optimal pH was 7.3. Optimal growth was observed when NaCl was omitted from the medium. Yeast extract stimulated growth and a vitamin supplement was required for growth.

The range of electron donors and acceptors used was similar for the four strains. The electron acceptors used were sulfate, sulfite and thiosulfate. Nitrate was not utilized. The compounds used as electron donors were as follows (mM): lactate (20), fumarate (10), acetate (10), formate (5), propionate (10), butyrate (10), succinate (10), H2/CO2 (80:20, v/v), glucose (20), fructose (20), ethanol (20), methanol (20), propanol (10), butanol (5) and isobutanol (5). Compounds not used were as follows (mM): isobutyrate (5), 3-chlorobenzoate (2), 2-propanol (5) and benzoate (5). Distinguishing features between the isolates were their μmax values on methanol (0.012–0.034 h⁻¹) and their tolerance for NaCl (0.7–2%). Table 1 shows characteristics of the representative strain (V21T) in comparison with those of its closest relatives.

Phospholipid fatty acid composition of bacterial cultures

Strain V21T contained almost exclusively only iso-C₁₅:₀ and iso-C₁₇:₀ as phospholipid fatty acids when cultured on methanol with sulfate (Table 2). This simple composition resembles that of Desulfotomaculum australicum (Love et al., 1993), a related strain (Table 2). The phospholipid fatty acid profiles of D. kuznetsovi and D. thermobenzoicum subsp. thermosyrupiticum were somewhat more complex, but iso-C₁₅:₀ and iso-C₁₇:₀ were also major compounds together with C₁₅:₀ and C₁₆:₀ (Table 2). The fatty acid composition of D. kuznetsovi reported here was rather different from that presented by Nazina et al. (1999). Possible explanations for this difference are that Nazina et al. (1999) studied total extractable fatty acids or, more likely, that they used cultures grown on propionate with sulfate. The substrate used may influence fatty acid compositions in bacteria especially when methanol (a C₁ compound) is used.
Phylogenetic analysis and taxonomic affiliation

For phylogenetic screening of the strains, their partial 16S rRNA gene sequences (790 bp) were compared and DNA–DNA homology determined. The 16S rRNA gene sequence similarity between the isolates was 93 %. Levels of sequence similarity of up to 95 % suggest that the phylogenetic distance is large enough for these strains to be designated as separate species (Stackebrandt & Goebel, 1994). However, these sequence similarities are based on partial sequences, and the DNA–DNA homology between the strains is high, ranging from 85 to 92 %. The G+C content of the DNA ranged from 48-3 to 48-7 mol%. High levels of physiological and phylogenetic similarity between the isolates justified the choice of one strain, i.e. strain V21T, as a representative of this group of isolates. For further study and taxonomic description, strain V21T was used as the type strain. A full 16S rRNA gene sequence of strain V21 T was obtained and compared with sequences of all other described Desulfotomaculum species. The topology of the resulting phylogenetic tree (Fig. 1) is in accordance with data published by others (Nilsen et al., 1996; Liu et al., 1997; Stackebrandt et al., 1997; Pikuta et al., 2000). In the phylogenetic tree, strain V21T consistently branches together with members of the genus Desulfotomaculum of subcluster IC. The closest relatives are D. kuznetsovi (93 % similarity), D. luciae (92 % similarity) and strain TPOSR (92 % similarity). DNA–DNA hybridization studies with strain V21T and D. kuznetsovi showed a homology value of 49 %, which justifies a novel species designation for strain V21T.

Table 1. Characteristics of strain V21T in comparison to those of its closest relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature optimum (°C)</td>
<td>60–65</td>
<td>NR</td>
<td>55–65</td>
<td>60</td>
</tr>
<tr>
<td>Temperature range (°C)</td>
<td>50–85</td>
<td>50–70</td>
<td>NR</td>
<td>48–65</td>
</tr>
<tr>
<td>Spore formation</td>
<td>Yes</td>
<td>Yes*</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Extremely heat-resistant spores</td>
<td>Yes</td>
<td>NT</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>NaCl requirement</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>49</td>
<td>51·4</td>
<td>55</td>
<td>48·3</td>
</tr>
<tr>
<td>Sulfide inhibition (initial conc.)</td>
<td>15</td>
<td>NR</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Growth factors required</td>
<td>No</td>
<td>No</td>
<td>NR</td>
<td>Vitamins</td>
</tr>
</tbody>
</table>

Electron donors:

- Glucose: −, −, −, +
- Fructose: −, −, −, +
- Acetate: +, −, +, +
- Propionate: +, −, +, +
- Butyrate: +, −, +, +
- Fumarate: +, NR, +, +
- Succinate: +, −, +, +
- Methanol: +, −, +, +
- Butanol: NR, NR, NR, +
- Isobutanol: NR, NR, NR, +

μmax on methanol (h⁻¹):

<table>
<thead>
<tr>
<th>Strain</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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<tbody>
<tr>
<td></td>
<td>0·033</td>
<td>−</td>
<td>0·03</td>
<td>0·017</td>
</tr>
</tbody>
</table>

Electron acceptors:

- Sulfite: −, −, −, +
- Nitrate: −, NR, −, −

*Not confirmed in this study.

Strain V21T is able to utilize methanol (Table 1), a characteristic it shares with D. kuznetsovi, Desulfotomaculum thermosapovorans, strain TPOSR and strain WW1 only. Methanol utilization by thermophilic sulfate-reducing organisms is a rare characteristic restricted to species of the genus Desulfotomaculum. Strain V21T can utilize fructose, like the moderate thermophiles Desulfotomaculum nigrificans and Desulfotomaculum geothermicum. Glucose utilization has not been described for thermophilic or mesophilic Desulfotomaculum strains and seems to be a unique property of strain V21T, though growth is weak. The organism with the closest sequence similarity to strain V21T is D. kuznetsovi. However, this organism produces extremely heat-resistant spores (H. P. Goorissen, unpublished data). The second closest relative, D. luciae, has a limited substrate range, is
unable to grow on methanol and does not use sulfite. Strain TPOS R, like D. kuznetsovii, produces extremely heat-resistant spores and does not grow on sugars. Moreover, the G + C content of its DNA is far higher than that of our organism. Strain TPOS R was isolated from an anaerobic bioreactor on propionate, whereas strain V21T was obtained from solfataric sediment.

Sulfite may be inhibitory for micro-organisms, and a sulfite concentration as low as 40 mg l\(^{-1}\) is inhibitory to sulfate-reducing organisms (Widdel \& Bak, 1992). Our batch-culture experiments with V21T, however, showed that sulfite concentrations up to 1-6 g l\(^{-1}\) did not inhibit sulfidogenesis with methanol (results not shown). The relatively high initial sulfide concentration used in our enrichment experiments may also have increased the selection pressure in favour of sulfate-reducing bacteria. Moreover, an initial sulfide concentration of 5 mM did not inhibit growth or sulfide production by our isolates. Initial sulfide concentrations above 15 mM totally inhibited growth.

On the basis of the physiological differences between strain V21T and all known related organisms, and the results of DNA–DNA hybridization studies, we propose that strain V21T represents a novel species of the genus Desulfotomaculum, namely Desulfotomaculum solfataricum.

### Description of Desulfotomaculum solfataricum sp. nov.

Desulfotomaculum solfataricum [sol.fa.ta’ri.cum. N.L. neut. adj. solfataricum pertaining to solfatares, derived from solfata (field of hot sulfur springs and fumaroles), referring to the original habitat of the organism).

Cells are straight rods of 1.5–2.5 μm in diameter and 3.5–5 μm in length, which occur singly and in pairs. Spores are spherical, central or subterminal and distend the cells. Gram stain is negative, but the cell wall structure is typical of Gram-positive micro-organisms. No gas vacuoles are observed. The following substrates are utilized as carbon and energy sources in the presence of sulfate: methanol, ethanol, propanol, butanol, isobutanol, H\(_2\)/CO\(_2\), acetate, formate, propionate, butyrate, lactate, fumarate, succinate, glucose and fructose. The electron acceptors used are sulfate, sulfite.

<table>
<thead>
<tr>
<th>Phospholipid fatty acid</th>
<th>Content (% total fatty acids) in:</th>
</tr>
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<tbody>
<tr>
<td>C14:0</td>
<td>2:1</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>55:3</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>1:0</td>
</tr>
<tr>
<td>C16:0</td>
<td>1:0</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>2:1</td>
</tr>
<tr>
<td>C16:0 9c</td>
<td>1:0</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>2:1</td>
</tr>
<tr>
<td>C16:0 9t</td>
<td>2:1</td>
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<tr>
<td>C16:0</td>
<td>0:1</td>
</tr>
<tr>
<td>C17:0</td>
<td>2:1</td>
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<tr>
<td>C17:0 9t</td>
<td>2:1</td>
</tr>
<tr>
<td>C18:0</td>
<td>2:1</td>
</tr>
<tr>
<td>C18:0 9t</td>
<td>2:1</td>
</tr>
<tr>
<td>C18:0</td>
<td>2:1</td>
</tr>
<tr>
<td>C19:0</td>
<td>2:1</td>
</tr>
</tbody>
</table>

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and thiosulfate. Nitrate is not utilized. Initial sulfide concentrations of up to 15 mM are tolerated. Grows fermentatively on lactate. Vitamins are required. The temperature range for growth is 48–65 °C; the optimum growth temperature is 60 °C. The pH range for growth is 6.4–7.9; the optimum pH is 7.3. The NaCl concentration range for growth is 0–1.5%; best growth occurs without NaCl. Phylogenetically, the species is a member of subcluster IC of the genus Desulfotomaculum.

The type strain of Desulfotomaculum solfatariicum is V21T (= DSM 14956T = CIP 107984T). Isolated from hot solfataric fields in north-east Iceland. The G+C content of its DNA is 48.3 mol%.

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