Thermophilic methanol utilization by sulfate reducing bacteria
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Endospores of thermophilic sulfate reducing bacteria survive extreme heat treatments


The ability of microorganisms to survive extreme environmental conditions is partly determined by their capacity to protect DNA from destructive chemical and physical conditions. Some bacteria form endospores that can resist extreme conditions — including pressure, extreme heat or cold, drought, starvation, most poisons, and UV-radiation. Spores may remain dormant for centuries and may even survive over geological time and through interstellar travel.

Here we report on the extreme heat resistance of spores of the thermophilic sulfate-reducing bacterium *Desulfotomaculum kuznetsovii*. About 10% of the spores of this organism survived a heat treatment at 140 °C for 15 min., which is unprecedented. This unusual characteristic is represented by a z-value (thermal inactivation coefficient) of 16.7 °C. The decimal reduction value at 120 °C is 4 hours, which is highest ever.
Introduction

Heat resistant spores are found among several species, mainly those belonging to the genera *Bacillus* and *Clostridium* (Hyung et al., 1983; Fernandez et al., 2001). Most of the research into heat resistance of spores has been done using mesophilic species in food industrial processes. Less attention has been paid to the heat resistance of spores formed by thermophilic bacteria. Spores from thermophilic bacteria are more heat resistant than spores from mesophilic species (Warth, 1978). Heat resistant spores from thermophilic anaerobes can be troublesome in research laboratories and routine autoclaving protocols of culture media and materials might be insufficient to inactivate bacterial spores. On the contrary, normal autoclaving procedures (20 min., 121 °C) may even activate and increase the apparent heat resistance of spores (Hyung et al., 1983; Byrer et al., 2000).

The first high decimal reduction value $D_T$, the incubation time at temperature $T$ necessary for a 90% decrease in the viability of the spores, for thermophilic species was reported in 1965 for *Clostridium thermoaceticum*, an acetogen with a $D_{124}$ of more than 72 minutes (Xezones et al., 1965). More recently, extremely heat resistant spores have been detected in other thermophilic *Clostridium* and *Moorella* species, i.e. *Cl. thermohydrosulfuricum* ($D_{120} = 11$ min.) (Hyung et al., 1983), *Cl. thermoautotrophicum* ($D_{120} = 70$ min.) (van Rijssel et al., 1992), and *Moorella thermoacetica* JW/DB2 and JW/DB4 ($D_{120} = 84$ min., 111 min., respectively) (Byrer et al., 2000). What determines the spore heat resistance is not known with certainty. Multiple factors are involved, such as the composition of the proteinaceous spore-coat (Henriques & Moran, 2000), the dipicolinic acid concentration of the spore-cortex (Beaman & Gerhardt, 1986), its thickness, and its $Ca^{2+}$ content, the dehydration and mineralization state of the spore (Popham et al., 1999), and specialized DNA-binding proteins termed $\alpha/\beta$ type small acid soluble spore proteins (SASP) (Setlow & Setlow, 1998).

Virtually no studies have been made of the heat resistance of spores of the thermophilic sulfate-reducing bacteria of the genus *Desulfotomaculum*, which are widespread in nature. Only for *D. nigrificans*, a moderate thermophile that was responsible for sulfur stinker spoilage in canned food products, a $D_{120} = 5.4$ min. was reported (Donnelly & Busta, 1980). We studied the metabolism of *Desulfotomaculum kuznetsovii*, a thermophilic, rod-shaped, spore-forming, sulfate-reducing bacterium isolated from a geothermal hot spring (Nazina et al., 1987). In continuous cultures, it appeared that sterilization procedures of even longer than two hours at 120 °C were insufficient to kill the spores of *D. kuznetsovii* in our fermentors. This observation led to the question whether this extremely heat resistant spore production is a typical phenomenon for all thermophilic *Desulfotomaculum* strains or is specific to *D. kuznetsovii*. All strains present in the IC and ID subclusters of the phylogenetic tree of the *Desulfotomaculum* main cluster were examined for the production of heat resistant spores.
Methods

Strains. The following strains were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany): *Desulfotomaculum kuznetsovii* (DSM 6115), *Desulfotomaculum thermoacetoxidans* (DSM 5813), *Desulfotomaculum thermobenzoicum* (DSM 6193), and *Desulfotomaculum thermocisternum* (DSM 10259). *Desulfotomaculum australicum* (AB33) and *Desulfotomaculum luciae* (SMCC W644) were kindly provided by Bharat Patel (Griffith University, Nathan, Queensland) and David Boone (Oregon Graduate Institute of Science and Technology, Portland Oregon) respectively. Strain TPOSR, strain WW1, and strain V21 were isolated in our laboratories.

Phylogenetic analysis. Sequences were downloaded from the Genbank Database and aligned using Clustal W. The 16S rDNA phylogenetic tree was constructed from a distance matrix based on the neighbor-joining method (Saitou & Nei, 1987) as implemented in the TREECON program (van de Peer & de Wachter, 1995). A manual correction method was applied and tree topology was re-examined by using bootstrap analysis (100 replicants, all bootstrap values ≥ 68).

Cultivation techniques. Anaerobic culture techniques were used throughout this study, with all media prepared as described in the catalog of strains of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (http://www.gbf.de/dsmz/media/media.htm). For the production and germination of the spores, substrates methanol (20 mM, filter sterilized) or lactate (20 mM, heat sterilized) were added from anoxic stock solutions. Incubations were carried out at 60 °C in 1 liter bottles filled with 100 ml medium for the spore-production experiments or in 16 ml tubes filled with 10 ml medium for the spore-germination experiments and most probable number counts.

Determination of D-values. From a 1 liter culture, cells and spores were harvested and concentrated 25-fold by centrifugation. Samples (2 ml) of this suspension were transferred to new anaerobic sterile tubes. Tubes were heated in an oil-bath for different times (1 to 40 hours) and different temperatures. After heat treatment, the spore suspensions were diluted in fresh anaerobic medium and incubated at 60 °C for most probable number counts. Decimal reduction values (D) were calculated from the best linear fit from y = ax + b, in which x is the incubation time (hours) and y is log N. N is the number of viable spores after heat treatment. Experiments were carried out in duplo.

Results

Determination of the heat resistance of *D. kuznetsovii* spores

Heat resistance of the spores of *D. kuznetsovii* was determined in duplicate culture suspensions (3·10⁹ viable cells per ml) at incubation temperatures of 120, 130, and 140 °C. The survival of viable spores after heat treatment was counted using the most probable number method. Survival curves of *D. kuznetsovii* spores are depicted in Fig. 4.1.

The decimal reduction value for *D. kuznetsovii* spores at 140° C is 15 min., an unprecedented value. At lower temperatures, the D-values are extremely high (D₁₃₀ = 79.2 min., D₁₂₀ = 240 min.) and this slow decrease of viability of the spores at higher temperatures is also illustrated by a high z-value i.e. 16.7 °C (Fig. 4.2).
Figure 4.1. Survival curve of *D. kuznetsovii* spores at different temperatures. Symbols: ◆, T = 120 °C; ■, T = 130 °C; ▲, T = 140 °C.

Figure 4.2. Thermal inactivation curve. Best fit through log decimal reduction values of *D. kuznetsovii* spores

Assuming first order kinetics for the inactivation of spores by heat, extrapolation of the thermal inactivation curve results in a $D_{100}$ of approximately 70 hours and a $D_{90}$ of more than 11 days. Altering the growth conditions could influence the apparent heat resistance of the spores. Omission of calcium from the sporulation medium reduced the $D_{120}$ to 87 min. This confirms the observation that bacterial spores accumulate calcium, which contribute to spore heat resistance, depending on the concentration in the growth medium (Popham *et al.*, 1999). The assumed relation between spore cortex/cytoplasm ratio and degree of heat resistance as postulated by Hyung *et al* (1983) could not be confirmed in our study. In *D. kuznetsovii* spores, this ratio is relatively low (1.7) in view of their extreme heat resistance.
Phylogenetic analysis of thermophilic *Desulfotomaculum* species

The complete 16S rDNA sequences of all *Desulfotomaculum* strains in subclusters IC and ID and some other strains that form heat resistant spores were compared. In the resulting phylogenetic tree (Fig. 4.3), species within the *Desulfotomaculum* subcluster IC are closely related, showing similarity values of 95-98%. The sequence similarity of the subclusters IC and ID is more than 90%.

![Phylogenetic tree](image)

**Figure 4.3.** Phylogenetic tree based on 16S rRNA gene sequence comparisons. The neighbor-joining tree was reconstructed from distance matrices; bootstrap values are not shown. Cluster designation according to Stackebrandt et al. (1997) The GenBank accession numbers for the organisms used in this analysis are *Desulfotomaculum kuznetsovii* AF009646; *Desulfotomaculum luciae* AF069293; *Desulfotomaculum nigrificans* X62176; *Desulfotomaculum thermoacetoxidans* Y11573; *Desulfotomaculum thermobenzoicum* L15628; *Desulfotomaculum thermocisternum* U33455; *Moorella thermoacetica* M59121; *Moorella thermoautotrophica* L09168; *Thermoanaerobacter siderophilus* AF120479; Strain TPOSR AF442686; Strain WW1 AF442687. *Bacillus methanolicus* X64465 S42879 served as outgroup.
**Determination of the heat resistance of spores of thermophilic *Desulfotomaculum* species**

Among all the studied strains, only strain TPOSR and strain WW1 produced extremely heat resistant spores, although their spores were considerably less heat resistant than *D. kuznetsovii* spores. The D_{120} value for WW1 is 60 min. and for TPOSR D_{120} is 114 min (Table 4.1). For both strains, tubes were positive after a heat treatment of 16 hours and negative after 20 hours. We were unable to obtain sporulating cultures of *D. australicum* and *D. luciae*. For *D. luciae*, it has been reported that cells were still viable after a 30 min. boiling procedure (Karnauchow et al., 1992). All other strains from subcluster IC and ID produced spores with D_{120}-values equal to or below 3 min. (Table 4.1).

**Table 4.1.** Highest reported D_{120}-values (values < 3 min. are not taken into account), combined with D_{120}-values of *Desulfotomaculum* strains from the 1C and 1D phylogenetic cluster

<table>
<thead>
<tr>
<th>Strain</th>
<th>D_{120} (min)</th>
<th>Sporecortex to cytoplasm ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus stearothermophilus</em></td>
<td>3</td>
<td>2.8</td>
<td>Aiba &amp; Humphrey, 1978</td>
</tr>
<tr>
<td><em>Clostridium difficile</em> 88</td>
<td>32.5</td>
<td>nr</td>
<td>Nakamura et al., 1985</td>
</tr>
<tr>
<td><em>Cl. thermoautotrophicum</em></td>
<td>70</td>
<td>1.4</td>
<td>van Rijssel et al., 1992</td>
</tr>
<tr>
<td><em>Cl. thermohydrosulfuricum</em> 39E</td>
<td>11</td>
<td>6.6</td>
<td>Hyung et al., 1983</td>
</tr>
<tr>
<td><em>Cl. thermosaccharolyticum</em></td>
<td>72.5</td>
<td>nr</td>
<td>Xezones et al., 1965</td>
</tr>
<tr>
<td><em>Desulfotomaculum nigricans</em></td>
<td>5.6</td>
<td>nr</td>
<td>Donnelly &amp; Busta, 1980</td>
</tr>
<tr>
<td><em>D. kuznetsovii</em></td>
<td>240</td>
<td>1.7a</td>
<td>This report</td>
</tr>
<tr>
<td><em>D. thermoacetoxidans</em></td>
<td>&lt; 3</td>
<td>nd</td>
<td>This report</td>
</tr>
<tr>
<td><em>D. thermobenzoicum</em></td>
<td>&lt; 3</td>
<td>nd</td>
<td>This report</td>
</tr>
<tr>
<td><em>D. thermocisternum</em></td>
<td>&lt; 3</td>
<td>nd</td>
<td>This report</td>
</tr>
<tr>
<td>Strain V21</td>
<td>&lt; 3</td>
<td>nd</td>
<td>This report</td>
</tr>
<tr>
<td>Strain TPOSR</td>
<td>114</td>
<td>nd</td>
<td>This report</td>
</tr>
<tr>
<td>Strain WW1</td>
<td>60</td>
<td>nd</td>
<td>This report</td>
</tr>
<tr>
<td><em>Moorella thermoacetica</em> JW/DB-4</td>
<td>111</td>
<td>5.97</td>
<td>Byrer, et al., 2000</td>
</tr>
</tbody>
</table>

nr, not reported; nd, not determined; a) determined as described previously (van Rijssel et al., 1992)

**Discussion**

The production of extremely heat resistant spores with a D_{140} of 15 min. is an exclusive feature of *D. kuznetsovii* and makes this strain unique, not only within the genus *Desulfotomaculum*, but among all spore-forming bacteria. To our knowledge, D_{140}-values higher than 7.9 sec. have not been demonstrated previously (Huemer et al., 1998). Highest D_{T} values reported are D_{120} values, found for thermophilic low G+C % gram-positive *Clostridium* and *Moorella* species (Table 4.1). On the basis of 16S rDNA sequence comparisons a relative high degree of relatedness between these species and the thermophilic *Desulfotomaculum* species exists (80-87%). *Desulfotomaculum* species have been isolated from anaerobic bioreactors and from environmental samples. In both classes, heat-sensitive spore producers are also present. This
indicates that the occurrence of *Desulfotomaculum* species with extremely heat resistant spores is not confined to a particular habitat.

The comparison of data published on the heat resistance of spores is complicated because no standard quantification and incubation protocols are available. Besides, environmental factors greatly affect microbial heat resistance. Composition of the growth medium (Payot *et al*., 1999; Casadei *et al*., 2001) sporulation temperature (Beaman & Gerhardt, 1986), the heating conditions and recovery medium (Coroller *et al*., 2001; Palop *et al*., 2000), and increased heating time (Byrer *et al*., 2000) influence the apparent heat resistance. Often a survival of boiling treatments is reported to indicate heat resistance, e.g. *D. thermoacetoxidans* survived 10 min. boiling, but did not survive 15 min. boiling (Min & Zinder, 1990); *Thermoanaerobacter siderophilus* survived a 90 min. boiling treatment (Slobodkin *et al*., 1999), and methanol utilizing *Desulfotomaculum* species survived a heat treatment at 131 ºC for 20 min. (Rosnes *et al*., 1991). Although these data might give a prediction of heat resistance, such observations are not comparable with D-values. In all our experiments, the same conditions have been applied. Therefore it is clear that the differences in heat resistance among the spores of different *Desulfotomaculum* species is an intrinsic property of the thermophilic *Desulfotomaculum* species.

**References**


