Clostridium thermosulfurogenes sp. nov., a New Thermophile that Produces Elemental Sulphur from Thiosulphate

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A new Clostridium species is described that was isolated from a thermal, volcanic, algal-bacterial community via selective enrichment procedures with pectin as energy source. Clostridium thermosulfurogenes sp. nov. deposits elemental sulphur on the cell surface and in the culture medium from thiosulphate transformation. This species stained Gram-negative, but electron micrographs revealed a double-layered wall without the presence of an outer membranous layer. Thin sections displayed numerous internal membranes and sulphur granules were not discernible. The organism was motile and formed distinctly swollen sporangia with terminal, white-refractile, spherical spores. The temperature range for growth was >35 °C and <75 °C, the pH range was between 4.0 and 7.5. The DNA base composition was 32.6 ± 0.04 mol% guanosine plus cytosine. Fermentable carbohydrates included pectin, starch, xylose, glucose, mannose, cellobiose, maltose, arabinose and sucrose. The doubling time on glucose or pectin was about 2 h. The production of ethanol, H₂/CO₂, acetate and lactate accounted for a balanced fermentation of glucose, whereas methanol and isopropanol were also produced during pectin fermentation. The taxonomic relationships of C. thermosulfurogenes to other thermophilic clostridia and its biological role in a thermal microbial community are discussed.

INTRODUCTION

Detailed studies on the biology of thermophilic bacteria have been initiated in the last ten years in order to learn more about their function in the environment and because of their potential process applications in biotechnology (Zeikus, 1979). The most progress on understanding thermophilic anaerobe diversity in nature has been made by examination of the bacterial species common to volcanic thermal spring ecosystems (Ben-Bassat & Zeikus, 1981; Wiegel & Ljungdahl, 1981; Zeikus, 1979; Zeikus et al., 1979, 1980). Biotecnological research with thermophilic anaerobes has been focused primarily on methane, ethanol or acetic acid production (Ljungdahl et al., 1981; Wang et al., 1978, 1979; Zeikus, 1979, 1980; Zeikus et al., 1981).

Doemel & Brock (1977) described the general properties of an algal-bacterial mat ecosystem in Yellowstone National Park, U.S.A., where biomass is produced and decomposed by thermophilic bacteria. Recently, the microbiology of anaerobic carbon mineralization was examined in the mat (Zeikus et al., 1980; Ben-Bassat & Zeikus, 1981). Anaerobic bacterial species active in biomass decomposition in this ecosystem included: Thermobacteroides acetethylicus, Thermoanaerobium brockii, Clostridium thermohydrosulfuricum and Methano bacterium thermoautotrophicum.

In the present report we describe the general morphological, cellular and metabolic characteristics of a new endospore-forming species that was repeatedly isolated from this environment by selective enrichment with pectin as the energy source for growth. The microbial properties detailed here enable distinction of this new species from C. thermocellum (Viljoen et al., 1926; Ng et al., 1977), C. thermohydrosulfuricum (Klaushofer & Parkkinnen, 1965), C. thermo-

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METHODS

Chemicals. All chemicals used were reagent grade and were obtained from either Mallinckrodt (Paris, Ky., U.S.A.) or Sigma. Pectin was a gift of Sunkist Growers (Corona, Calif., U.S.A.). Agar, tryptone and yeast extract were obtained from Difco. All gases were obtained from Matheson (Joliet, Ill., U.S.A.) and were purified free of oxygen by passage over heated (370 °C) copper filings.

Inocula and bacterial strains. Two thermophilic strains were enriched from a 60 °C site in the Octopus Spring algal–bacterial mat in Yellowstone National Park, Wyo., U.S.A., employing anaerobic procedures described previously (Zeikus et al., 1979, 1980). One strain was enriched in October, 1978, and a second strain in August, 1980. Both isolates had identical properties. Data presented in the results section are for type strain 4B.

Culture techniques and media. Anaerobic procedures used for cell cultivation and media preparation were described previously (Zeikus et al., 1980; Schink et al., 1981). The low phosphate buffered basal medium (LPBB medium) described by Zeikus et al. (1979) was used with a N2/CO2 (95:5) gas phase. When needed, carbohydrates (0.5%, w/v), tryptone (1%, w/v), or yeast extract (0.3%, w/v) were added by syringe from separately autoclaved concentrates. The medium of Ng et al. (1977) was used to examine cellulose fermentation, and Medium 77 (Postgate, 1963) was used to test sulphate reduction, but glucose was substituted for lactate. Routine cell maintenance and experimental analysis was performed in anaerobic culture tubes (23 ml volume, 18 × 142 mm) obtained from Bellco Glass Co. (Vineland, N.J., U.S.A.) containing 10 ml of medium, and sealed with black rubber bungs. Prior to inoculation, culture medium was reduced by the addition of sodium sulphide (0.05%, w/v final concentration). Test medium for quantification of thiosulphate reduction contained LPBB medium with 0.3% yeast extract; 0.05% FeSO4, 7H2O, 0.02% sodium thioglycollate, and 0.02% ascorbic acid, but no sodium sulphide was added. Cultures were routinely incubated without shaking at 60 °C unless indicated in the text. For isolation of colonies in anaerobic roll tubes LPBB medium was supplemented with 0.5% glucose, 0.1% yeast extract and 2% purified agar. Large quantities of cells were grown at pH 7.0 with mixing in a MicroFerm fermenter (New Brunswick) containing 12 l of LPBB medium, 0.5% glucose and 0.1% yeast extract.

Cellular characterization. A Carl Zeiss photomicroscope was used for phase-contrast and bright-field observations including determination of cell size. Agar-coated glass slides were utilized to obtain long filaments in focus for photomicroscopy.

The methods used for preparing cells for thin sectioning and electron microscopic examination were as described by Kellenberger et al. (1958). Cells were prefixed for 2 min in 0.1% osmium tetroxide, washed in acetate/veronal buffer, suspended in agar, and then fixed overnight in 1% osmium tetroxide. All preparations were examined with a Siemens 101 electron microscope by Dr A. Ryter, Institut Pasteur, Paris, France.

DNA was isolated and purified from lysozyme-treated cells by the method of Marmur (1961). DNA base compositions were calculated according to the method of DeLey (1970) in 0.015 M NaCl and 0.0015 M-trisodium citrate as determined in a Gilford Model 250 spectrophotometer equipped with a model 2527 thermoprogrammer. Escherichia coli DNA VIII (lot no. 57C-6830, Sigma) served as standard. DNA composition reported represents the mean of four separate determinations. The G + C content of the E. coli standard was 53.9 ± 1.0%.

Cytochromes were identified in air versus dithionite-reduced difference spectra of cell extracts analysed with a Beckman model 25 scanning spectrophotometer. Cell extracts were prepared by sonicating a suspension of 1 g wet weight cells in 5 ml 50 mm-potassium phosphate buffer and retaining the supernatant after centrifugation at 10000 g for 15 min in a Sorvall centrifuge. Protein was determined in extracts by the Lowry method.

Growth and metabolic characterization. Growth was determined by measuring the increase in turbidity at 660 nm. Absorbance was estimated directly by insertion of the anaerobic culture tubes into a Spectronic 20 spectrophotometer (Bausch & Lomb).

Fermentation metabolites were measured directly in liquid or gas samples removed from the culture tubes by syringe. Alcohols and acids were measured by gas chromatography, with a flame ionization detector, as described by Zeikus et al. (1979). All gases were quantified by the gas chromatography–thermal conductivity detection methods of Nelson & Zeikus (1974). Lactate was measured spectrophotometrically by the D- or L-lactate dehydrogenase assay procedure of Bergmeyer (1965). Glucose consumption was determined by reducing-sugar analysis with dinitrophenyl salicylic acid (Miller et al., 1960).

Protein was determined by the biuret method modified for sulphur-containing solutions as described by Szarkowska & Klingenberg (1963). This method uses a control for non-protein dependent colour formation by destruction of the colour complex via addition of potassium cyanide. Thioulsphate, sulphite, and sulphide were determined in cell-free culture fluids by the methods of Pachmayr (1960). Sulphur was determined in culture fluid by extraction with carbon disulphide and spectrophotometric analysis as described by Fliermanns & Brock (1972).
RESULTS

Isolation and cultivation

The organism was selectively enriched from the Octopus Spring algal–bacterial mat by serial dilution of the source inoculum in LPBB medium that contained 1-0% tryptone, 0-3% yeast extract and 0-5% pectin. The last positive tube (i.e. 10–3 dilution) displaying growth and gas production was repeatedly transferred on LPBB medium containing 0-1% yeast extract and pectin. The organism was isolated by serial dilution in agar roll tubes with LPBB, 0-1% yeast extract and 0-5% glucose medium followed by transfer of single colonies to homologous liquid medium.

Single colonies were light yellow, 0-5-1-5 mm in diameter, and had a fluffy, brush-like appearance. The organism formed large colonies on agar plates incubated in an anaerobic chamber. Stock cultures maintained viability when held at room temperature or at 4 °C for two weeks. Permanent stocks were kept for more than six months by freezing anaerobic cell suspensions in 20% glycerol at −80 °C.

Cellular properties

The morphological features of *C. thermosulfurogenes* varied considerably with the age of cultures and the growth medium used (Fig. 1). Exponential phase cells grown on glucose LPBB/yeast extract medium were actively motile by peritrichous flagella, phase-dark, and stained Gram-negative. Stationary phase cells were more phase-light in appearance and contained dark granules. Cells existed as rods that varied considerably in length depending on the medium (i.e. as single short rods of 2 μm to filamentous chains greater than 20 μm). Sporulation was never observed in glucose media but was detected in late-stationary phase cultures grown with xylose or pectin as the energy source. Sporulation was especially favoured in LPBB medium containing 0-1% yeast extract, 0-2% xylose and 1-0% MOPS buffer. Sporulating cells were on the average larger than vegetative cells and formed swollen, terminal, spherical spores (Fig. 1 b). Only a few sporulating cells became distinctly phase-bright and white in old cultures; free spores were rare.

Most notably, the addition of sodium thiosulphate at 20 mM resulted in very turbid cultures (i.e. $A_{660} > 2-0$) that contained a white–yellow precipitate at the end of growth. Microscopic analysis of these cultures revealed the presence of yellow elemental sulphur granules (Fig. 1 c, d).

Electron microscopic analysis of *C. thermosulfurogenes* in thin section revealed some architectural features that were not typical of other clostridia (Fig. 2). Notably, internal membranes were very numerous and often appeared vesicular. The cell wall was thin and poorly discernible, but was double-layered as in most clostridia. Unlike many other Gram-negative staining bacteria no outer wall membrane layer was present. Large, electron-dense cytoplasmic granules were common in cells. All spores were spherical and swollen, but most appeared abortive because of the absence of an electron-transparent spore cortex. Thin sections of cells grown with thiosulphate did not display structures discernible as sulphur inclusions.

DNA isolated from *C. thermosulfurogenes* had a base composition of 32-6 ± 0-04 mol% guanosine plus cytosine. Difference spectra of crude cell extracts did not display absorbance bands corresponding to cytochromes. Cells lacked catalase. Cell proliferation was not detectable in the presence of: cycloserine, penicillin, streptomycin, tetracycline or chloramphenicol [each at 100 μg (ml culture)−1]; sodium azide (500 μg ml−1); sodium chloride (2%, w/v); and $O_2$ (21% in the culture headspace).

Growth and metabolic properties

The optimum temperature for growth of *C. thermosulfurogenes* was near 60 °C, the maximum was below 75 °C and the minimum above 35 °C (Fig. 3). The growth rate and yield (i.e. turbidity) of *C. thermosulfurogenes* varied with the nutrient sources supplied (Fig. 4). The growth rate and yield was lowest on mineral medium with ammonium as nitrogen source and highest in complex medium with tryptone and yeast extract. The organism was repeatedly transferred (20 times) on glucose and mineral medium. In complex medium the cell doubling time on polymeric
pectin (2.1 h) was only slightly higher than that on glucose (1.9 h). The optimum pH for growth on glucose was 5.5-6.5; growth was not observed below 4.0 or above 7.6.

_Clostridium thermosulfurogenes_ fermented a wide variety of energy sources including: L-arabinose, cellobiose, galactose, glucose, inositol, mannitol, maltose, melibiose, rhamnose, sucrose, trehalose, D-xylose, starch, pectin, polygalacturonic acid, amygdalin, aesculin and salicin. Cellulose, arabinogalactan, galacturonate, citrate, pyruvate, lactate, tartrate, lactose,
Clostridium thermosulfurogenes sp. nov.

Fig. 2. Electron micrographs of thin sections of C. thermosulfurogenes grown on LPBB, 0.3% (w/v) xylose, 0.2% (w/v) yeast extract medium. (a) Vegetative cells illustrating internal membranes. (b) Sporulating cultures showing a swollen sporangium (S) and vegetative cell (V). Arrows point to 'double-track' appearing wall layers. (c) Free spore illustrating electron-transparent spore cortex and external coat layers. The bar marker represents 1 μm in (a) and (b), and 0.5 μm in (c).

melezitose, raffinose, d-ribose, sorbitol, methanol and glycerol were not fermented. The organism liquefied gelatin, but neither produced indol, acetyl-methylcarbinol or hydrogen sulphide nor reduced sulphate or nitrate.

The main products of carbohydrate fermentation by C. thermosulfurogenes were H₂/CO₂, ethanol, acetate and lactate (Table 1). Notably, on pectin as energy source, both methanol and
isopropanol were formed. Considerably more ethanol and lactate were produced on glucose than on polygalacturonate which is a more oxidized substrate. The fermentation balance calculated on glucose was 242 glucose → 231 H₂ + 207 ethanol + 152 acetate + 113 lactate + 317 CO₂. This accounted for a carbon recovery of 95% and an oxidation/reduction index of 0.98.

The dependence of fermentation product formation on growth is shown in Fig. 5. All end products increased in response to growth and glucose consumption. However, both glucose consumption and end product formation continued after growth ceased. Growth appeared uncoupled by the high proton concentration (pH < 4.5) in the medium. Growth on glucose at pH
Table 1. Relationship between energy source and fermentation products formed by C. thermosulfurogenes

All experiments were performed in anaerobic culture tubes that contained 10 ml LPBB medium, 0.3% (w/v) yeast extract and 0.5% (w/v) carbohydrate. Products were analysed after 24 h incubation at 60 °C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrogen</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Acetate</th>
<th>Lactate</th>
<th>Isopropanol</th>
<th>CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>231</td>
<td>–</td>
<td>207</td>
<td>152</td>
<td>113</td>
<td>–</td>
<td>317</td>
</tr>
<tr>
<td>Pectin</td>
<td>316</td>
<td>113</td>
<td>22</td>
<td>190</td>
<td>28</td>
<td>12</td>
<td>212</td>
</tr>
<tr>
<td>Polygalacturonate</td>
<td>239</td>
<td>–</td>
<td>37</td>
<td>265</td>
<td>23</td>
<td>–</td>
<td>195</td>
</tr>
<tr>
<td>Xylose</td>
<td>173</td>
<td>–</td>
<td>220</td>
<td>175</td>
<td>19</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Arabinose</td>
<td>168</td>
<td>–</td>
<td>222</td>
<td>194</td>
<td>113</td>
<td>–</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not determined.

Table 2. Inorganic sulphur transformations of C. thermosulfurogenes

All experiments were performed in anaerobic culture tubes containing 10 ml LPBB medium, 0.1% (w/v) yeast extract and 0.5% (w/v) glucose, but with a FeSO₄/thiosulphate/ascorbate reducing agent in lieu of Na₂S. Products were analysed after 24 h incubation at 60 °C. Controls were uninoculated but treated the same way as other tubes.

<table>
<thead>
<tr>
<th>Additions to medium (mM)</th>
<th>Growth</th>
<th>Sulphur metabolites (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Turbidity (A₆₅₀)</td>
<td>Protein (mg ml⁻¹)</td>
</tr>
<tr>
<td>None</td>
<td>0.54</td>
<td>0.17</td>
</tr>
<tr>
<td>Na₂S₂O₃ 12.5</td>
<td>0.96</td>
<td>0.17</td>
</tr>
<tr>
<td>25</td>
<td>1.2</td>
<td>0.17</td>
</tr>
<tr>
<td>25 (control)</td>
<td>0.14</td>
<td>ND</td>
</tr>
<tr>
<td>Na₂SO₃ 20</td>
<td>0.36</td>
<td>0.08</td>
</tr>
</tbody>
</table>

ND, Not determined.

7·0 was not inhibited by normal end-product concentrations of ethanol, acetate, lactate or by three atmospheres of H₂/CO₂.

Table 2 demonstrates the type of thiosulphate transformation shown by C. thermosulfurogenes. In the absence of thiosulphate, growth occurred without formation of inorganic sulphur metabolites; however, growth was not as good as in the medium with cysteine or sodium sulphide as reducing agent. Notably, the addition of increasing amounts of thiosulphate increased the culture turbidity but not the total cell protein formed. Thiosulphate was converted to elemental sulphur, but neither sulphite nor sulphide was produced. The addition of sulphite inhibited growth, but it was not converted to elemental sulphur.

DISCUSSION

The discovery of C. thermosulfurogenes extends the diversity both of known anaerobic thermophiles and of the described microbial sulphur transformation reactions. Inorganic sulphur compounds are transformed into elemental sulphur by a limited number of microbial groups. The deposition of elemental sulphur within cells or culture media appears limited to anaerobic phototrophic bacteria (Pfenning, 1967) and several aerobic sulphide-oxidizing bacterial genera (Buchanan & Gibbons, 1974; Schedel & Trüper, 1980). However, these kinds of bacteria utilize reduced sulphur compounds as electron donors. Clostridium thermosulfurogenes is the first fermentative chemoorganotrophic bacterium reported to form sulphur from
thiosulphate. This transformation is especially novel because no sulphide or sulphite was
detected during the conversion of thiosulphate to sulphur. The biochemical basis and
physiological significance of this transformation remains a mystery. It is worth noting here that
another member of the Yellowstone algal–bacterial, thermal community deposits yellow
 elemental sulphur: the phototroph Chloroflexus aurantiacus (Madigan & Brock, 1975).

The presence of C. thermosulfurogenes appears of importance to the algal–bacterial mat
ecosystem. The chemical composition of the major particulate fraction of primary production in
this 55–65 °C ecosystem (i.e. microbial cell wall layers) is not cellulose but, rather, carbohydrate
and peptide-glycan polymers because the main phototrophs are Synechococcus lividus and
Chloroflexus aurantiacus (Doemel & Brock, 1977; Brock, 1978). Although other fermentative
anaerobes such as C. thermohydrosulfuricum, T. acetocethylicus, T. brockii and M. thermoauto-
trophicum have been isolated from this environment, none of these strains can degrade pectin.
There are no specific data about the pectin content of thermophilic algae, but it seems probable
that Synechococcus lividus contains pectin in its sheath material as do other cyanobacterial
species (Desikachary, 1959). Thus, pectin decomposition is of importance to prevent organic
matter accumulation in this ecosystem where primary production and organic mineralization
appear well-coupled (Doemel & Brock, 1977). Clostridium thermosulfurogenes appeared as the
prevalent pectinolytic anaerobe and was present at \( \geq 10^3 \) cells (ml algal mat \(^{-1} \)). However, this
species is present in much lower numbers than total anaerobes \( \geq 10^8 \) cells (ml algal mat \(^{-1} \); 
Zeikus et al., 1980). Nonetheless, the number of pectin degraders in the algal mat is equivalent to
that found in eutrophic lake sediments \( (10^2-10^5 \text{ cells ml}^{-1} ) \) that receive cyanobacteria as the
major carbon input (Schink & Zeikus, 1982).

Clostridium thermosulfurogenes was clearly distinguishable from the other described
thermophilic saccharolytic Clostridium species (Buchanan & Gibbons, 1974). Like other
Clostridium species examined by electron microscopy (Sleytr & Glauert, 1976), it contained a
double-layered wall architecture, but did not possess an outer wall membrane layer common to
some Gram-negative staining anaerobes (e.g. Bacteroides or Desulfovibrio). Since it produced
ethanol as the major soluble reduced end product of growth, and not butyrate or acetate, it is not
similar to C. thermosaccharolyticum (Hsu & Ordal, 1970) or C. thermoaceticum (Fontaine et al.,
1942). Motility, spherical spore formation and absence of cellulose fermentation distinguishes it
from C. thermocellum (McBee, 1948; Ng et al., 1977). It is most similar to C. thermohydrosulphuri-
cum in substrate utilization range and fermentation end products, but it forms elemental sulphur
from thiosulphate rather than H\(_2\)S (Matteuzzi et al., 1978). Other significant differences
between the Octopus Spring strains of C. thermosulfurogenes and C. thermohydrosulfuricum
(Zeikus et al., 1980) include: lack of growth inhibition by hydrogen, morphological features of
sporogenesis, and the ability to liquefy gelatin and to ferment pectin. The ability of C.
thermosulfurogenes to form methanol as a major fermentation product of pectin is a consequence
of pectin methylesterase activity and the absence of methanol consumption by described
pectinolytic bacteria (Schink & Zeikus, 1980).

Clostridium thermosulfurogenes may have some catabolic features of biotechnological interest.
As an ethanol- or lactate-producing thermophile it grows at lower pH values than C.
thermocellum, T. brockii and T. acetocethylicus (Zeikus et al., 1981). Notably, the ethanol/
lactate ratio increased dramatically during growth on pentoses. This species can hydrolyse a
variety of polymers including pectin, starch and gelatin. Clostridium thermosulfurogenes would
appear to have very active pectinases because its growth rate on pectin is nearly equivalent to
that on glucose.

Clostridium thermosulfurogenes sp. nov.

ther.mo.sul.fu.ro'ge.nes. Gr. n. thermos heat; L. n. sulfur sulphur; Gr. n. gennao to produce.
thermosulfurogenes producing sulphur in heat.

Cellular characteristics. Straight rods 0.5–2 \( \mu \)m. Stains Gram-negative; exponential phase
cells motile by peritrichous flagellation. Forms long filaments and deposits yellow elemental
sulphur on cells and in the medium when grown with thiosulphate. Swollen, white-refractile,
spherical endospores formed. No outer wall membranous layer present in thin sections. Agar
Clostridium thermosulfurogenes sp. nov.

Embedded colonies are fluffy, 0.5-1.5 mm in diameter, and not pigmented. DNA base composition of 32.6 (± 0.04) mol% G + C. Cytochromes undetectable; catalase negative.

Growth characteristics. Optimum temperature for growth is ≥ 60°C, maximum 75°C, minimum 35°C. pH range for growth: optimum 5.5-6.5, minimum >4.0, maximum <7.6. Obligate thermophile and anaerobe. Complete growth inhibition by penicillin, streptomycin, cycloserine, tetracycline, chloramphenicol (each at 100 μg ml⁻¹), sodium azide (500 μg ml⁻¹) or O₂ (0.203 × 10⁵ Pa).

Metabolic characteristics. Chemoorganotroph. Utilizes a wide variety of carbohydrates as energy sources including pectin, arabinose, cellobiose, polygalacturonate, rhamnose, amygdalin, salicin, inositol, mannitol, xylose, galactose, glucose, mannose, maltose, starch, melibiose, sucrose, trehalose or aesculin. No growth on H₂/CO₂, lactose, cellulose, tartrate, lactate, pyruvate, methanol or glycerol. Fermentation products of glucose are ethanol, H₂, CO₂, lactate and acetate. Methanol and isopropanol formed on pectin. Sulphate, sulphite and nitrate not reduced.

Habitat. Algal–bacterial mat ecosystems associated with thermal, volcanic springs. Type strain 4B. This strain was isolated from Octopus Spring, Yellowstone National Park, U.S.A. The strain has been deposited in both the Deutsche Sammlung von Mikroorganismen (DSM 2229) and the American Type Culture Collection, Rockville, Md., U.S.A. (ATCC 33743).

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