Clostridium sulfidigenes sp. nov., a mesophilic, proteolytic, thiosulfate- and sulfur-reducing bacterium isolated from pond sediment

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A novel strictly anaerobic, endospore-forming, rod-shaped, Gram-positive bacterium, designated strain SGB2^T, was isolated from a mixed culture from a pond sediment during screening for sulfate-reducing bacteria capable of degrading cyanophycin (CGP). In this study, the taxonomic characterization of this mesophilic, proteolytic *Clostridium* isolate and the role which it, and its phylogenetic relatives, may play in peptide degradation and in the sulfur cycle are reported. Strain SGB2^T was a commensal strain, utilizing CGP degradation products produced by other micro-organisms. Cells were motile until sporulation, forming oval, terminal spores that swell the cells. It showed optimum growth at 34 °C, pH 6.6 and in the absence of NaCl. Strain SGB2^T utilized proteinaceous compounds such as peptone, Casamino acids, gelatin and trypticase soy, in addition to several amino acids and pyruvate. Utilization of many of these compounds was enhanced in the presence of thiosulfate. The isolate was unable to use any of the carbohydrates or alcohols investigated or CGP as carbon and energy sources. Thiosulfate and elemental sulfur were used as terminal electron acceptors. Phylogenetic analysis revealed that strain SGB2^T belongs to the low-G + C-containing *Clostridiales* group. It exhibited 99% 16S rRNA gene sequence similarity to its closest relatives *Clostridium thiosulfatireducens* Lup21^T and *Clostridium subterminale* DSM 6970^T. DNA–DNA hybridization values with these two strains were 39.4 and 42.1%, respectively. Based on phenotypic, genotypic and phylogenetic characteristics, we conclude that the isolate represents a novel species of the genus *Clostridium*, *Clostridium sulfidigenes* sp. nov. The type strain is SGB2^T (=DSM 18982^T =ATCC BAA-1538^T).

While several *Clostridium* species are well known for their role in the nitrogen cycle as N₂-fixing bacteria (Purushothaman *et al.*, 1981), sulfide toxicity in several habitats such as rice-field soils has been attributed exclusively to sulfate-reducing bacteria that also reduce thiosulfate and sulfur. Thus, the establishment of the taxon *Desulfotomaculum* resulted in the reclassification of *Clostridium nigrificans* and a few other bacteria as members of this new genus, with the sulfate reducer *Desulfotomaculum nigrificans* (previously *C. nigrificans*) as the type species (Campbell & Postgate, 1965). This led to the distinct definition of clostridia as being strictly anaerobic, Gram-positive, endospore formers that are unable to carry out dissimilatory sulfate reduction (Cato *et al.*, 1986; Collins *et al.*, 1994). However, some exceptional cases have also been reported, such as asporogenous mutants that may appear and proliferate, different degrees of aerotolerance

known for several species and mesophilic clostridia that show a transient ability to reduce sulfate (Campbell & Postgate, 1965; Hippe *et al.*, 1992).

It was found recently that thiosulfate-reducing clostridia unable to reduce sulfate may also contribute to the formation of sulfide from inorganic sulfur compounds (Escoffier *et al.*, 1998, 2001), e.g. the saccharolytic, thermophilic bacteria *Clostridium thermohydrosulfuricum*, *Clostridium thermosaccharolyticum* (Hollaus & Sleytr, 1972) and *Clostridium thermosulfurigenes* (Schink & Zeikus, 1983), which were subsequently reclassified and now belong to the genera *Thermoanaerobacter* and *Thermoanaerobacterium* (Lee *et al.*, 1993). Only two mesophilic, proteolytic bacteria, *Clostridium peptidivorans* (Mechichi *et al.*, 2000) and *Clostridium thiosulfatireducens* (Hernández-Eugenio *et al.*, 2002), are able to reduce thiosulfate and are currently members of the genus *Clostridium*. In this study, an isolate belonging to the genus *Clostridium*, strain SGB2^T, which is a strictly anaerobic, spore-forming bacterium isolated from an

Abbreviation: CGP, cyanophycin.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SGB2^T is EF199998.

anaerobic consortium (pond sediment samples, Schloßgraben, Münster, Germany) as a member of a cyanophycin (CGP)-degrading consortium, is reported. The polymer CGP consists of equimolar amounts of aspartic acid and arginine (Simon & Weathers, 1976) and functions as a temporary nitrogen, energy and possibly carbon reserve in cyanobacteria. CGP is degraded by intracellular cyanophycinases or by extracellular cyanophycinases of bacteria unable to synthesize CGP (Obst *et al.*, 2005). Strain SGB2^T itself was unable to use CGP as a carbon and energy source, but occurred as a commensal micro-organism in the consortium and utilized CGP degradation products (Asp–Arg dipeptides) (Sallam & Steinbüchel, 2008).

The enrichment of axenic cultures, DNA extraction and sequencing of the 16S rRNA gene of strain SGB2^T were reported recently by Sallam & Steinbüchel (2008). Accordingly, strain SGB2^T was assigned to the genus *Clostridium*, with *Clostridium subterminale* DSM 6970^T and *C. thiosulfatireducens* Lup21^T as its closest phylogenetic relatives (99 % 16S rRNA gene sequence similarity for both). However, analysis of the G + C content and DNA–DNA hybridizations (both performed at the DSMZ) showed that strain SGB2^T had a slightly higher G + C content (32.3 mol%) than these two strains (28 mol% and 31.4 mol%, respectively) and DNA–DNA hybridization values were only 42.1 and 39.4 %, respectively, which indicated that strain SGB2^T is clearly not a strain of *C. subterminale* or *C. thiosulfatireducens*.

The next closest phylogenetic relative to strain SGB2^T was '*Clostridium tunisiense*' DSM 15206 (96 % 16S rRNA gene sequence similarity), whereas *C. peptidivorans* TMC4^T showed much lower similarity to strain SGB2^T (93 %). Using only a trimmed part (1434 bp) of the 16S rRNA gene sequence of strain SGB2^T and the available sequences for related strains in the NCBI database, a phylogenetic tree was generated (Fig. 1). This tree shows the relationship between strain SGB2^T, *C. subterminale* DSM 6970^T, the

sulfur-reducer '*C. tunisiense*' DSM 15206 and related species reported to reduce thiosulfate. DNA G + C content analysis and the phylogenetic tree showed that strain SGB2^T is a member of the low-G + C-containing Gram-positive bacteria and represents a member of cluster I of the order *Clostridiales* (Collins *et al.*, 1994; Stackebrandt & Rainey, 1997).

Growth studies were performed in duplicate under anaerobic conditions in Hungate tubes containing basal medium (Sallam & Steinbüchel, 2008) with 0.5 % (w/v) proteose peptone (Difco) and were inoculated from similar precultures. Cells of strain SGB2^T multiplied only in an oxygen-free anaerobic atmosphere and at temperatures between 18 and 48 °C, with optimum growth at 34 °C, which is lower than its taxonomic relatives. Strain SGB2^T could grow at pH 5.5–9.0, with optimum growth at pH 6.6. Optimum growth occurred in the absence of NaCl; however, retarded growth occurred up to an NaCl concentration of 20 g l⁻¹. Additionally, strain SGB2^T differed from its closest relatives in several other criteria including reduction of electron acceptors and the range of utilizable amino acids (Table 1).

C. thiosulfatireducens DSM 13105^T and *C. peptidivorans* DSM 12505^T were used as reference strains to investigate the use of inorganic electron acceptors by strain SGB2^T. This was determined photometrically by the increase in growth (turbidity) and H₂S production as described by Sallam & Steinbüchel (2008). Table 1 shows the characteristics of strain SGB2^T and other related strains regarding reduction of inorganic sulfur compounds. Notably, sulfate was not reduced by axenic cultures of strain SGB2^T except, non-reproducibly, in a few experimental repetitions (two of 18 independent repetitions) whereas, in co-cultivations with other isolates from the same mixed consortium, sulfate reduction by strain SGB2^T was stable over several repetitions (Sallam & Steinbüchel, 2008). In contrast, growth of strain SGB2^T in the presence of thiosulfate consistently started after 1 day of incubation, yielding a maximum OD₅₇₈ of 0.7 after 10 days and producing 2.6 mM H₂S.

Growth and H₂S production by strain SGB2^T on the utilized substrates is shown in Fig. 2. The presence of thiosulfate improved substrate utilization as revealed by growth to higher OD, in particular on proteose peptone (Difco) (22.8 %), Casamino acids (10 %), trypticase soy (17.2 %), L-arginine (21.5 %), L-ornithine (14.5 %) and L-citrulline (9.2 %). Relatively large amounts of H₂S were produced in the presence of thiosulfate, especially on proteose peptone (4.8 mM), Casamino acids (3.8 mM), trypticase soy (5.5 mM), gelatin (8.0 mM), L-arginine (4.0 mM), L-valine (3.4 mM) and L-cysteine (3.7 mM) (Fig. 2). In the absence of thiosulfate, only negligible H₂S production was observed.

Fermentation products of the utilized substrates were measured by HPLC (LaChrom Elite HPLC; VWR-Hitachi) as described by Sallam & Steinbüchel (2008). The main

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the estimated phylogenetic relationships of *Clostridium sulfidigenes* sp. nov. SGB2^T and closely related species. Accession numbers are given in parentheses. Bootstrap values are shown as percentages of 100 replicates. Bar, 2 % sequence divergence.

Table 1. Comparison between the characteristics of strain SGB2^T and its closest phylogenetic relatives

Strains: 1, strain SGB2^T (data from this study); 2, *C. subterminale* DSM 6970^T (unless indicated, data from Elsdén & Hilton, 1979; Cato *et al.*, 1986; Hippe *et al.*, 1992); 3, *C. thiosulfatireducens* DSM 13105^T (unless indicated, data from Hernández-Eugenio *et al.*, 2002); 4, *C. peptidivorans* DSM 12505^T (unless indicated, data from Mechichi *et al.*, 2000); 5, '*C. tunisiense*' DSM 15206 (data from Thabet *et al.*, 2004). Studies on the utilization of electron acceptors and substrates were performed in liquid basal medium containing 0.5 % (w/v) Casamino acids for electron acceptors, or 1 g yeast extract l⁻¹, 20 mM sodium thiosulfate and no cysteine for substrate utilization. The following concentrations were used in electron acceptor tests: 20 mM sodium sulfate, 20 mM sodium thiosulfate and 2 % (w/v) elemental sulfur. Substrate concentrations were: 10 mM for amino acids, 5 g l⁻¹ for peptides and 20 mM for carbohydrates and organic acids. All experiments were performed in Hungate tubes and incubated at 34 °C for at least 15 days. Additionally, L-proline, L-isoleucine, L-serine, peptone, Casamino acids, gelatin and CGP dipeptides were utilized by all strains. The following substrates were tested and were not utilized by any of the strains: lactose, glucose, galactose, mannose, arabinose, xylose, cellobiose, ribose, maltose, sucrose, fructose, lactate, acetate, succinate, ethanol, 1-propanol and CGP. ND, No data available.

Characteristic	1	2	3	4	5
Source	Pond sediment	Human infections	Cheese factory wastewater	Olive mill wastewater	Olive mill wastewater
Growth temperature					
Range (°C)	18–48	25–45	18–45	20–42	18–43
Optimum (°C)	34	37	37	37	37
Growth pH					
Range	5.5–9.0	5.4–9.3	6.0–9.8	6.0–9.0	5.5–8.7
Optimum	6.6	7.5 ^a *	7.4	7	7.8
NaCl concentration for growth					
Range (g l ⁻¹)	0–20	0–80	0–60	0–40	0–30
Optimum (g l ⁻¹)	0	0–10	0	0	0–15
DNA G + C content (mol%)	32.3	28	31.4	31	32.6
Thiosulfate reduction	+	– ^b	+ ^c	+ ^c	–
Sulfur reduction	+	ND	+ ^c	– ^c	+
Utilization of:					
L-Citrulline	+	ND	+ ^c	ND	ND
L-Ornithine	+	ND	+ ^c	ND	+
L-Aspartate	–	ND	– ^c	–	ND
L-Arginine	+	+	– ^c	+	ND
L-Lysine	–	+	+ ^c	+	+
L-Alanine	+	+ ^a	+ ^c	–	–
L-Histidine	–	+ ^a	+ ^c	+	+
L-Leucine	+	+	+ ^c	–	+
L-Methionine	+	+ ^a	+ ^c	+	–
L-Threonine	+	–	+ ^c	–	+
L-Phenylalanine	+	+	+ ^c	–	ND
L-Valine	+	+	+ ^c	–	+
L-Cysteine	+	ND	– ^c	+	+
L-Glutamate	–	+	– ^c	–	ND
Glycine	–	+	– ^c	–	ND
DL-Tryptophan	–	+	– ^c	–	–
L-Tyrosine	–	+	– ^c	–	ND
Trypticase soy	+	ND	+ ^c	ND	+
Pyruvate	+	+	+ ^c	ND	+

*Data from: a, Thabet *et al.* (2004); b, Hernández-Eugenio *et al.* (2002); c, this study.

fermentation end-products from Casamino acids, trypticase soy and gelatin were acetate, propionate, valerate and butyrate. Peptone was converted mainly to acetate, succinate, propionate and valerate, whereas yeast extract was fermented to acetate and ethanol. The main fermentation end product detected from citrulline, ornithine,

alanine, threonine, serine and cysteine was acetate. Utilization of arginine produced acetate and ethanol, whereas utilization of methionine and phenylalanine produced mainly propionate. Valine was converted to butyrate, leucine to valerate, and pyruvate to acetate and ethanol.

Fig. 2. Growth and H₂S production of strain SGB2^T in the presence of various substrates. Cultivation experiments were done in basal medium containing 1 g yeast extract l⁻¹ and 20 mM sodium thiosulfate, in addition to the indicated substrate at concentrations of 10 mM for amino acids, 5 g l⁻¹ for peptides and 20 mM for pyruvate, incubated at 34 °C for 6 days. Shaded bars show growth (change in OD₅₇₈ compared with control tubes); open bars show H₂S production (mM). Data shown represent means and standard deviations from duplicate cultivation tubes.

Based on the phenotypic, genotypic and phylogenetic characteristics mentioned above and in Table 1, the novel isolate was designated the type strain of a novel species of the genus *Clostridium*; *Clostridium sulfidigenes* sp. nov. This study provides another example, in addition to previous observations, that strain SGB2^T and other proteolytic clostridium strains probably play a significant role in the sulfur cycle and in the degradation of peptides, proteins and amino acids in different environments. They are greatly affected by the presence of different forms of inorganic and organic sulfur compounds. In addition, the increasing number of reports on *Clostridium* species with such special traits extends the known diversity of this genus.

Description of *Clostridium sulfidigenes* sp. nov.

Clostridium sulfidigenes (sul.fi.di'ge.nes. N.L. n. *sulfidum* sulfide; N.L. neut. suff. *-genes* producing from Gr. v. *gennaio* to produce; N.L. neut. part. adj. *sulfidigenes* sulfide-producing).

Cells are rods, about 3–6 µm long and 0.5 µm in diameter, occurring as single cells or in pairs, and are motile until sporulation. Subterminal to terminal spores are formed. Gram staining is positive. Colonies appearing after 48 h of incubation on peptone at 34 °C and after only 24 h on yeast extract are round with regular margins, translucent on peptone and yellowish brown on yeast extract. Optimum growth temperature is 34 °C, with growth at 18–48 °C. Optimum pH for growth is 6.6 and growth occurs at pH 5.5–9.0. Optimum growth occurs in the absence of NaCl; however, concentrations up to 20 g NaCl l⁻¹ are tolerated. The following substrates are used as carbon and energy sources: peptone, Casamino acids, gelatin, trypticase soy, L-citrulline, L-ornithine, L-arginine, L-alanine, L-leucine, L-isoleucine, L-methionine, L-threonine, L-serine, L-phenylalanine, L-valine, L-cysteine and pyruvate. The following substrates are not used: L-proline, L-aspartate, L-lysine, L-histidine, L-glutamate, glycine, DL-tryptophan, L-tyrosine, lactose, glucose, galactose, man-

nose, arabinose, xylose, cellobiose, ribose, maltose, sucrose, fructose, lactate, acetate, succinate, ethanol and 1-propanol. The main fermentation end products from Casamino acids, trypticase soy and gelatin are acetate, propionate, valerate and butyrate. Peptone is converted mainly to acetate, succinate, propionate and valerate, whereas yeast extract is converted to acetate and ethanol. The main fermentation end product detected from citrulline, ornithine, alanine, threonine, serine and cysteine is acetate; from arginine utilization, end-products are acetate and ethanol, and from methionine and phenylalanine, the end-product is mainly propionate. Valine is converted to butyrate, leucine to valerate, and pyruvate to acetate and ethanol. Uses thiosulfate and sulfur, but not sulfite, nitrate or nitrite as electron acceptors. Thiosulfate improves the utilization of proteose peptone (Difco), Casamino acids, trypticase soy, L-arginine, L-ornithine and L-citrulline.

The type strain is SGB2^T (=DSM 18982^T =ATCC BAA-1538^T), isolated from pond sediment in Münster, Germany. Adverse effects on humans and animals by strain SGB2^T are not known, but cannot be excluded. Cautious handling and autoclaving of cultures before disposal is recommended. Strain SGB2^T has a DNA G+C content of 32.3 mol% (HPLC).

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