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Clostridium thermoautotrophicum Species Novum, a Thermophile Producing Acetate from Molecular Hydrogen and Carbon Dioxide

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Abstract. Fourteen strains of a thermophilic, rod-shaped, peritrichously flagellated *Clostridium* species were isolated from various mud and soil samples. Round to slightly oval spores were formed in terminal position. The isolates were obligate anaerobes and grew chemolithotrophically with H_2 plus CO_2 as well as chemoorganotrophically with fructose, glucose, glycerate, or methanol. Under both conditions, acetate was the only organic fermentation product formed in significant amounts. The pH optimum for growth was 5.7; the marginal temperatures for growth were T_{min}, 36°C; T_{opt}, 56–60°C; and T_{max}, 69/70°C. The DNA contained 53–55 mol% guanine plus cytosine. The isolated strains form a new clostridial species; the name *Clostridium thermoautotrophicum* is proposed.

Thirty-five years ago, Wieringa [16,17] isolated and described the first organism producing acetate from H_2 plus CO_2 according to the following equation:

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$$

The organism was named *Clostridium aceticum*; it was lost after 1948, but an original spore preparation was discovered recently, and Braun, Mayer, and Gottschalk [4] revived the spores and redescribed this organism. In addition, the formation of acetate from H_2 and CO_2 by sporeforming bacteria [2,12,14] and by a nonsporeformer [2] was reported in recent years. An organism closely related to *C. aceticum* is *C. formicoaceticum* [1]. Both organisms carry out homoacetate fermentations and convert, for example, 1 mol of hexoses to almost 3 mol of acetate. *C. formicoaceticum,* however, is unable to utilize molecular hydrogen and to form acetate from H_2 plus CO_2. It shares this inability with a thermophilic bacterium of the homoacetate fermentation type, *C. thermoaceticum* [7]. The question was whether a thermophilic clostridium exists that, like the mesophilic *C. aceticum,* is capable of producing acetate from H_2 plus CO_2.

In this communication, the isolation of 14 strains of such a species is described, and the establishment of a new species, *Clostridium thermoautotrophicum*, is suggested.

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Materials and Methods

Organisms. *Clostridium thermoaceticum* was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, DSM 521. The new isolates are listed in Table 1.

Culture and isolation techniques. Media preparation, enrichment, isolation, and cultivation of the organisms were done as described by Braun, Schoberth, and Gottschalk [5] except that the incubation temperature for growth was 60°C.

Growth on various carbon sources and under various conditions was examined in Hungate tubes (Bellco Glass, Inc., Vineland, New Jersey, 15 × 125 mm). The compounds were neutralized if necessary, sterilized by filtration through membrane filters (0.2 μm; Sartorius, Göttingen) and added to the basal medium to give a final concentration of 0.5% (wt/vol). Growth was followed by measuring the increase in the optical density at 650 nm (Bausch & Lomb Spectronic 80 spectrophotometer). The temperature dependence of the doubling time was determined using a temperature gradient incubator (Model TN-3; Tokyo Kagaku Sangyo Co. Ltd., Tokyo). The tests for the taxonomic identification were performed following the instructions of the *Anaerobe Laboratory Manual* [8].

Glucose was determined enzymatically using the Merckotest no. 3389 (Merck, Darmstadt).

The fermentation products were determined gas chromatographically (Perkin-Elmer 900 gas chromatograph; 2-mm-by-2-m glass column containing Porapak QS, 80–100 mesh; a temperature program from 90 to 200°C with an increase of 1°C per min was used).

DNA isolation. The DNA was isolated by the method described by Marmur [10]; in addition, a proteinase K step (Boehringer, Mannheim) was included. The base composition of the DNA was determined from its thermal denaturation temperature [11]. The hyperchromicity obtained was between 35 and 40%.

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Table 1. Sources for the isolation of the various strains of *Clostridium thermoautotrophicum*.

Strain	Source	Temperature and pH value of sample collected[a]
JW 701/3	Mud and water from hot spring at "White Creek", Yellowstone National Park, Wyoming, USA	47–50°C, pH 8.5
JW 701/5		
JW 702	Water sample from hot spring at "White Creek", Yellowstone National Park	70°C, pH 8.3
JW 703	Water and sediment from hot spring "Dragons Mouth", Yellowstone National Park	55°C, pH 5.5
JW 704	Water and mud from Oconee River, Georgia, USA	Ambient, pH 6.8
JW 705	Water and mud from a lake in Levin'scher Park, Göttingen, Federal Republic of Germany	Ambient, pH 6.7–7.0^{++}
JW 706	Mud from a lake in "Schiller Wiesen", Göttingen	Ambient, pH 6.5–6.8^{++}
JW 707	Mud from river Leine, Göttingen	Ambient, pH 7.3–7.8^{++}
JW 708	Mud from a spring in a wood (Södderich), near Göttingen	15°C, pH 6.9–7.8^{++}
JW 710	Mud from the Bay of Naples, Italy	Ambient, pH 7.7
JW 715	Wet soil from Solfatara Hawaii (Big Island)	45°C, pH—
JW 716	As source of JW 715	60°C, pH—
JW 717	Wet soil from tropical forest near Honolulu, Hawaii	Ambient, pH 5.9
JW 718	Wet soil from tropical forest on Hawaii (Big Island)	Ambient, pH 6.1
KIVU	Mud from Lake Kiwu, Zaire, Africa	—, —

[a] —, Not determined; $^{++}$, variation occurred in parallel small samples taken at different spots and depth.

The DNA hybridization was measured from renaturation rates according to De Ley, Cattoir, and Reynaerts [6]; a Gilford 250 spectrophotometer equipped with a thermo-cuvette (Gilford Instrument Laboratories, Oberlin, Ohio) was used.

Electron microscopy. Cells were negatively stained with uranyl acetate (4%; pH 5) according to Beuscher, Mayer, and Gottschalk [3]. Electron micrographs were taken with a Philips EM 301 electron microscope.

Results

Isolation of thermophilic acetogens. Samples from various locations (Table 1) were collected in N_2-flushed, sterile, 100-ml Sovirel flasks. In the laboratory, the samples were flushed with oxygen-free N_2 for at least 5 min. Aliquots of 3–10 g were transferred into 25 or 50 ml of a prereduced chemolithotrophic medium in 100- or 250-ml bottles under an atmosphere of 67% H_2 plus 33% CO_2. To suppress methane formation, bromoethanesulfonate was added to give a final concentration of 3 μM. The samples were incubated at 60°C, and the gas consumption was determined by the syringe method of Paynter and Hungate [13]. From all enrichments which had consumed more than 2 ml gas mixture per milliliter culture in 10 days, subcultures were made which were again transferred after 10 days. Fourteen pure cultures of acetate-producing bacteria (Table 1) were isolated from the second series of subcultures by repeated streaking of 1:10 dilutions on agar. The flat bottle method described by Braun, Schoberth, and Gottschalk [5] was employed for this. All isolates capable of producing acetate as the only product from H_2 and CO_2 were sporeformers. Their cells resembled each other in size, shape, and location of spores.

Counts of chemolithotrophic acetate-forming thermophiles. In samples from the Göttingen vicinity, the number of thermophiles producing acetate from H_2 plus CO_2 was determined. A direct plating of appropriate dilutions of the samples on agar-containing media was employed as described for the mesophilic bacteria by Braun, Schoberth, and Gottschalk [5]. No acid-producing colonies were obtained, presumably because of the predominance of fast-growing methanogens present in the samples. However, if the agar contained 3 μM bromoethanesulfonate, colonies producing acid were found sporadically in flasks containing 0.1 ml of the undiluted samples or 0.1 ml of suspensions of 2 g soil in 10 ml buffer. A preincubation of the samples for 5 min at 100°C, to activate presumed spores, did not lead to higher counts. Therefore, 1 g of these samples contained less than 100 bacteria capable of producing acetate from H_2 plus CO_2 at 60°C. This was significantly less than the number found at 37°C [5], but agreed with results on the enumeration of thermophiles utilizing other substrates [15].

Colony and cell morphology. The surface colonies of the isolated strains were smooth, almost round, flat, and tannish white (nonpigmented). They grew up to about 1 (with H_2 plus CO_2) and 3 mm (with glucose)

in diameter during 10 days. Colonies might turn brownish after 10 days of incubation. Cells of the early logarithmic growth phase exhibited a positive Gram reaction, whereas cells of later stages gave a negative reaction. The vegetative cells were rods of 3–6 μm in length and 0.8–1 μm in width (Fig. 1a,e,h). Young cells were slightly motile by 3–8 peritrichously inserted flagella, which were 8–14 μm long and 140–150 Å thick (Fig. 1h,i). The flagella hook consisted of 6–8 ringlike structures (Fig. 1i,k). The surface layer of the outer cell wall showed a tetragonal structure. The layer consisted of subunits of about 112 Å in diameter (Fig. 1i). Spores were frequently formed on agar-containing media and during the early stationary growth phase in liquid media. Under chemolithotrophic growth conditions as well as with formate or with methanol as carbon source, the sporulation was almost 100% (Fig. 1d,g). The spores were located in the swollen, terminal region of the cells (1.5–3 μm in diameter) and were released during the stationary growth phase (Fig. 1b–d). The free spores were round to slightly oval and 1–2.5 μm in diameter.

Up to four pili of the 50-to-60-Å type per cell were found on negatively stained cells. The cell wall is of the LL-diaminopimelic acid type (O. Kandler, Munich; personal communication).

Nutritional requirements and substrates for growth. Yeast extract (0.01%, wt/vol) was required for chemolithotrophic as well as for heterotrophic growth and could not be substituted for by a mixture of vitamins (vitamin solution according to [18]). In the absence of hexoses or carbon dioxide but in the presence of yeast extract (1%, wt/vol) (with and without H_2), very little growth was obtained, indicating that yeast extract did not serve as a sole carbon and energy source. Phosphate concentrations above 50 mM were inhibitory or caused a prolonged lag phase up to 3 weeks. This was mainly observed with methanol or CO_2 as carbon sources.

Gas mixtures of 33% CO_2 plus 67% H_2 were converted by the acetogenic thermophiles to stoichiometric amounts of acetate (Table 2). In addition, the organisms could utilize various sugars and acids and methanol (Table 3). With all substrates, acetate was the only organic fermentation product found in significant amounts. The amount of acetate produced by strain JW 701/3 from glucose is given in Table 2. It can be seen that 2.5 mol of acetate per mol of glucose was formed. Thus, these bacteria carry out a homoacetate fermentation. The ability to grow on H_2 plus CO_2 or on methanol was not lost after 5 subsequent transfers on glucose; however, for reproducible chemolithotrophic growth it was necessary to activate the spores by a heat treatment (5 min at 100°C) or to use cells of the early logarithmic growth phase as inoculum.

Temperature and pH range for growth. At pH 6.0, all strains isolated grew well at 60°C but not at 37°C. The marginal temperatures determined for strain JW 701/3, KIVU, and JW 708 were T_{min}, 36°C; T_{opt}, 56–60°C; and T_{max}, 69/70°C. In respect to the doubling time, these strains had a pH optimum around pH 5.7. No growth was observed if the initial pH value of the medium was below pH 4.7 or above pH 7.5. With glycerate as carbon source, the doubling time was 2 h at 60°C and at pH 5.6–5.8. Under chemolithotrophic conditions, the doubling time was 8 h with strain JW 701/3 at 58°C and a pH between 5.6 and 5.9.

DNA base composition. The base composition was 53.8 and 54.8 mol% guanine plus cytosine for strain JW 701/3 and strain KIVU, respectively. Hybridization experiments with DNA from *Clostridium thermoautotrophicum* strain JW 701/3 or strain KIVU and DNA from *C. thermoaceticum* revealed 48.5 and 52% homology, whereas the homology was 98% between the DNAs of the two chemolithotrophic strains.

Discussion

After *Clostridium aceticum* [4,16,17] and *Acetobacterium woodii* [2], a new species, *Clostridium thermoautotrophicum*, was isolated, representing the third bacterium capable of growing at the expense of acetate formation from H_2 plus CO_2. After *C. thermoaceticum*, it is the second thermophilic organism carrying out a homoacetate fermentation of organic compounds [9]. Unlike *C. thermoaceticum*, of which only one strain had been isolated from horse manure [7], several strains of the new chemolithotrophic thermophile were obtained from various sources and locations in the United States, Africa, and Europe. *C. thermoautotrophicum* seems to be widespread and its distribution is not restricted to thermal sources.

C. thermoautotrophicum and *C. thermoaceticum* seem to be closely related to one another, as indicated by a considerable homology of their DNAs (about 50%). However, differences are also apparent: *C. thermoaceticum* is unable to grow chemolithotrophically or with methanol. In addition, cells of the new isolates are wider (0.9 μm in width compared to 0.4 μm [7]); their terminal spores are located in big swollen sporangia, whereas spore-containing cells of *C. thermoaceticum* are only slightly swollen if

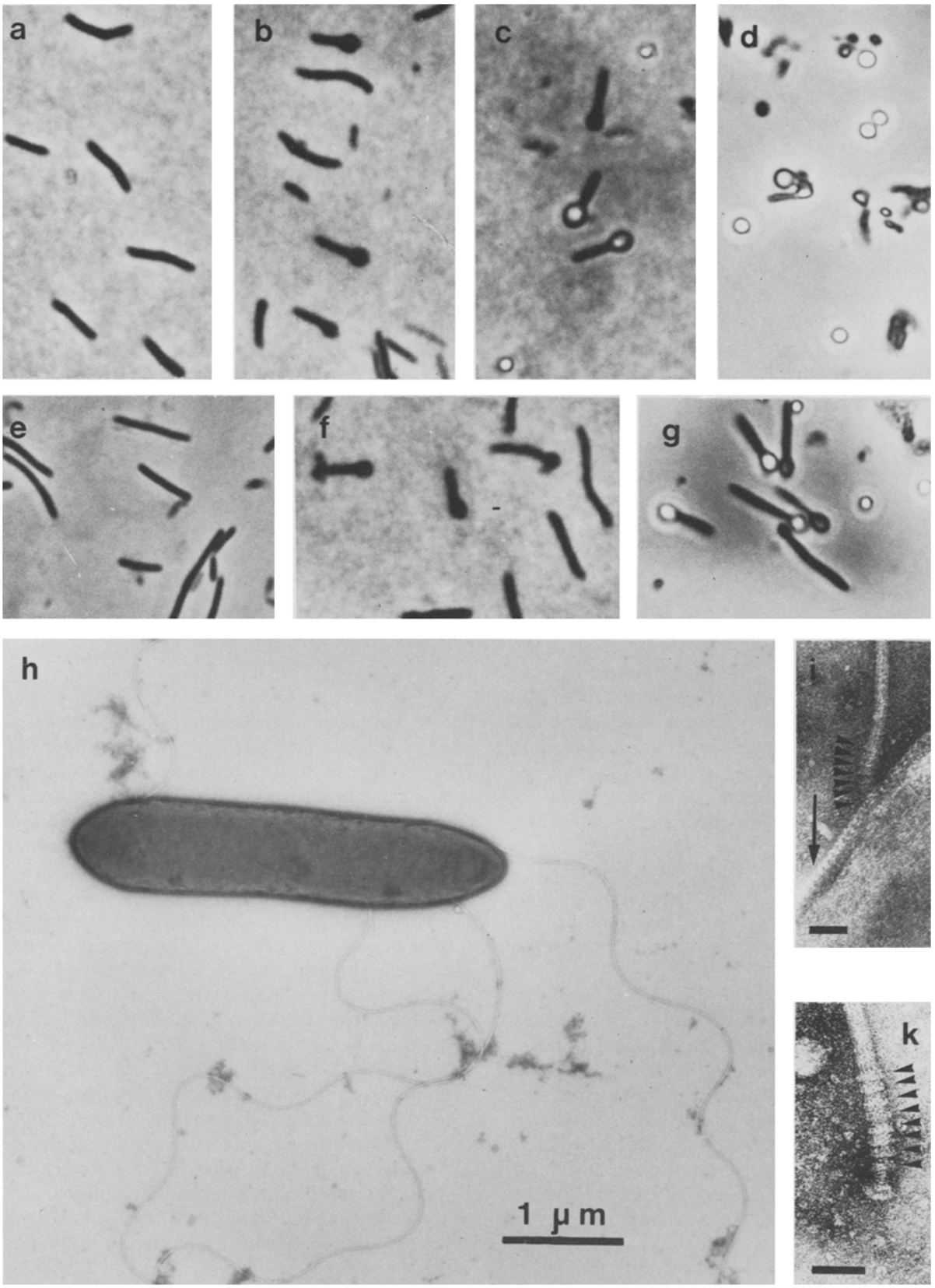
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Table 2. Amounts of acetate produced from H_2 plus CO_2 or glucose by *Clostridium thermoautotrophicum*.

Strain	Substrate consumed		Acetate formed (μmol/ml culture)	
	Substrate	μmol/ml culture	Calculated	Found
JW 701/3	$H_2 + CO_2$	448	74.7	78.2
	Glucose	26	—	65.3
KIVU	$H_2 + CO_2$	388	64.0	60.5

Table 3. Utilization of substrates by various strains of *Clostridum thermoautotrophicum*.[a]

Strain substrate	JW 701/3	JW 701a/5	JW 703	JW 704	JW 708	JW 716	JW 718	KIVU
H_2 plus CO_2	+	+	+	+	+	+	+	+
Formate	+	+	+	+	+	+	+	+
D-Glucose	++	++	(+)	++	++	++	++	++
D-Fructose	++	++	++	++	+	++	++	++
D-Galactose	++	++	+	++	(+)	++	(+)	++
D-Arabinose	−	+	−	−	−	−	−	−
D-Mannose	−	−	−	−	−	−	+	+
D-Xylose	−	+	−	−	−	−	+	−
D-Lactose	−	+	−	−	−	−	−	−
D-Maltose	−	+	−	−	−	−	−	−
Glycerate	++	++	++	++	++	++	++	++
Lactate	(+)	−	−	−	+	−	−	−
Methanol	+	(+)	+	+	+	+	+	+

[a] ++, Fast growth, optical density at 600 nm (Bausch & Lomb) above 1.0 with 0.5% (wt/vol) carbon source after 4 days; +, slow growth or low optical density; (+), poor utilization, very slow increase in optical density (0.1–0.3 in 10 days); −, no growth and no change in the pH value.

at all. Spores are formed by *C. thermoautotrophicum* more frequently than by *C. thermoaceticum*. The range of growth temperature is larger for *C. thermoautotrophicum* as compared with the range for *C. thermoaceticum*. These differences, especially in respect to chemolithotrophic growth, justify the proposal to establish a new species.

Description of *Clostridium thermoautotrophicum* species novum

Clostridium thermoautotrophicum sp. nov.; ther.mo.au.to.tro.phi.cum. Gr. adj. *thermus* hot, Gr. adv. *autos* self, Gr. n. *trophus* food, *thermoautotrophicum* indicating that the organism grows at elevated temperatures and uses carbon dioxide as its principal carbon source for growth.

Morphology. Vegetative cells are rod shaped, measuring 3–6 μm by 0.8–1.0 μm. The Gram-stain reaction of cells from the early logarithmic growth phase is positive, from later growth phases negative. Cells are slightly motile by 3–8 peritrichously inserted flagella (140–150 Å thick; 8–15 μm long). The surface layer of the outer cell wall shows a tetragonal structure, and consists of subunits with about 112 Å in diameter.

Spores. Round to slightly oval spores are formed at the polar cell region in oval sporangia (2–3 μm in diameter). The spores were normally released during the stationary growth phase and they measure 1–2.5 μm in diameter.

Cell wall. LL-Diaminopimelic acid type.

Colony characteristics. Surface colonies are tannish-white, circular convex, and smooth; colonies may turn brownish with time.

DNA base composition. The DNA of *C. thermoautotrophicum* contains 53–55 mol% guanine plus cytosine.

Fig. 1. Light microscopic and electron microscopic observations of *Clostridium thermoautotrophicum*. (a–g) Phase-contrast light microscopy; ×1,600. (a–d) Strain JW 701/3 under chemolithotrophic growth conditions at 60°C. (a) Cells of logarithmic growth phase (2nd day), (b) cells with pre-spores (4th day), (c) sporulated cells and free spores (5th day), (d) free spores (10th day). (e–g) Strain JW 708 grown with methanol as carbon source. (a) Cells of the logarithmic growth phase (36 h), (b) cells with pre-spores (50 h), (c) spore-containing cells and free spores (72 h). (h–k) Electron microscopy. (h) Single cell of JW 701/3 with flagella. (i) Higher magnification of insertion point of flagellum, of flagellum hook with ringlike structures (arrowheads) and of subunits of the outer cell envelope layer (arrow). (k) Separated flagellum hook with ringlike structure (arrowheads). Bars represent 50 nm.

Culture conditions. Strict anaerobe; T_{min}, 36°C; T_{opt}, 55–58°C; T_{max}, 69/70°C; pH range for growth 4.5–7.6, pH optimum around 5.7.

Nutritional characteristics. The organism converts 4 mol of molecular hydrogen and 2 mol of carbon dioxide to 1 mol of acetate. Acetate is also the only main organic fermentation product during growth on glucose, fructose, galactose, methanol, glycerate, or formate; only some strains can utilize arabinose, xylose, mannose, maltose, D-lactose, or lactate. The following compounds could not be used as carbon and energy source: D-ribose, D-fucose, D-mannose, sucrose, D-raffinose, starch, *m*-erythritol, xylitol, dulcitol, sorbitol, ethanol, ethyleneglycol, propanol, *m*-inositol, isopropanol, glycerol, malate, glutamate, pyruvate, succinate, citrate, tartrate, Casamino Acids, hippurate, nicotinic acid, and cellulose.

Habitat. Widespread but in low numbers; present in mud and wet soils from Africa, Europe, North America, and Hawaii. Not restricted to locations with elevated temperature.

Type strain. *C. thermoautotrophicum* JW 701/3 is designated as the type strain and is deposited in the German Collection of Microorganisms in Göttingen under the number DSM 1974.

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