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Fermentation of Inulin by *Clostridium thermosuccinogenes* sp. nov., a Thermophilic Anaerobic Bacterium Isolated from Various Habitats

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Four closely related strains of thermophilic bacteria were isolated via enrichment in batch and continuous culture with inulin as the sole source of carbon and energy by using inoculations from various sources. These new strains were isolated from beet pulp from a sugar refinery, soil around a Jerusalem artichoke, fresh cow manure, and mud from a tropical pond in a botanical garden. The cells of this novel species of strictly anaerobic, gram-positive bacteria were rod shaped and nonmotile. Growth on inulin was possible between 40 and 65°C, with optimum growth at 58°C. All strains were capable of fermenting a large number of sugars. Formate, acetate, ethanol, lactate, H_2, and succinate were the main organic fermentation products after growth on fructose, glucose, or inulin. Synthesis of inulinase in batch culture closely paralleled growth, and the enzyme was almost completely cell bound. Strain IC is described as the type strain of a new species, *Clostridium thermosuccinogenes* sp. nov., with a G+C content of 35.9 mol%.

Pure cultures and anaerobic consortia involved in the degradation of biopolymers at moderate temperatures (i.e., 20 to 45°C) have been studied quite extensively (17, 25). In contrast, our knowledge of microorganisms growing at more elevated temperatures is still very limited (11, 43, 54). Detailed studies on pure cultures and anaerobic microbial communities growing at the expense of sugar polymers will greatly enlarge our insight into the mineralization of organic carbon both at moderate and at elevated temperatures (12, 17, 25, 30, 41). Moreover, such knowledge is absolutely essential for the successful exploitation of microorganisms in various fermentation processes (7, 13, 20–22, 28).

Thus far, most studies of anaerobic polysaccharide degradation have focused on glucose-containing polymers such as starch and (hemi)cellulose. Another polysaccharide, inulin, which is a linear β-1-2-linked fructose polymer with one terminal glucose and is widespread in terrestrial ecosystems (2), has received little attention in microbiological studies. For this reason we have chosen this polyfructose as a model substrate for the study of thermophilic, polysaccharide-fermenting anaerobes. This natural storage polymer is found in several plant families of temperate regions, including the Compositae, Liliaceae, and Gramineae (2).

Biotechnologically, inulin is of great interest, as it may serve as a relatively cheap and abundant substrate for the microbiological production of high-fructose syrups (15, 49). It may also be used for ethanol (36) and acetone-butanol (27) fermentation. In addition, it may serve as a source of various sugar oligosaccharides of medical and dietary interest (53).

In most microbiological studies of the use of inulin as a potential substrate in the fermentation industry, yeasts, fungi, and mesophilic bacteria have been used (15, 49). A limited number of thermophilic, aerobic bacilli with inulinase activity have been described in some detail (1). However, almost no information on the properties and the potential applicability of strictly anaerobic species and the enzymes involved in the thermophilic breakdown of inulin is available. For this reason, a number of enrichments were performed in the present study, yielding various inulin-hydrolyzing organisms.

In this paper, we present detailed information about four closely related inulin-hydrolyzing thermophilic bacterial strains, all of which were shown to belong to the genus *Clostridium*. To account for the unusual ability of these clostridia to produce significant quantities of succinate as a fermentation product, a new species, "*Clostridium thermosuccinogenes*," is proposed.

(A patent application concerning the isolation and use of the thermophilic clostridia described herein for production of succinic acid has been filed [U.S. patent 493,408].)

MATERIALS AND METHODS

Origin of inulin-degrading bacteria. The bacteria were enriched from fresh cow manure, beet pulp from the extraction column of a sugar refinery, soil immediately around Jerusalem artichoke tubers, and sediment (50°C) from a tropical pond in the botanical garden of the University of Groningen.

Media and cultivation conditions. The bacteria were routinely cultivated at 58°C at a pH of 7.0 ± 0.2 in a basal medium containing the following components (in grams per liter): NaCl (1.2), $MgCl_2 \cdot 6H_2O$ (0.4), KCl (0.3), $CaCl_2 \cdot 2H_2O$ (0.15), NH_4Cl (0.27), KH_2PO_4 (0.205), Na_2SO_4 (0.1), $NaHCO_3$ (2.52), $Na_2S \cdot 9H_2O$ (0.15), yeast extract (0.025), Casamino Acids (0.025), and resazurin (0.001), as well as trace elements solution Sl 6^+ (35) (1 ml $liter^{-1}$) and vitamin solution (19) (1 ml $liter^{-1}$). The mineral medium was autoclaved under a gas phase of N_2-CO_2 (80:20, vol/vol) or N_2 (100%) with a bicarbonate buffer or a phosphate buffer, respectively. For growth under an atmosphere of 100% N_2, $NaHCO_3$ was added up to a concentration of 0.42 g $liter^{-1}$ (5 mM). NaH_2PO_4 (2.06 g $liter^{-1}$) and Na_2HPO_4 (2.66 g $liter^{-1}$) were used to buffer the medium at a neutral pH. The following stock solutions, aliquots of which were added aseptically to the mineral medium with plastic syringes, were sterilized separately: $NaHCO_3$ (84 g $liter^{-1}$), NaH_2PO_4 (68.6 g $liter^{-1}$), Na_2HPO_4 (157.3 g $liter^{-1}$), yeast extract (50 g $liter^{-1}$), Casamino Acids (40 g

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liter^{-1}) and vitamin solution. The vitamin solution and the trace elements solution were filter sterilized, and the other components were autoclaved for 20 min at 120°C. Inulin was sterilized for 30 min at 110°C to prevent decomposition.

For routine purity testing on anaerobic agar plates, nutrient-rich M17 Broth (Difco, East Molesey, United Kingdom) was amended with agar (2%, wt/vol) and inulin (0.15%, wt/vol).

Enrichments. The bacteria were enriched in batch culture by using 600-ml bottles filled with 250 ml of bicarbonate-buffered basal medium and inulin (1.5 g liter^{-1}) as the sole source of carbon and energy. The same medium was used for enrichments in continuous culture. Pure cultures were obtained after dilution in screw-cap culture tubes (16 by 125 mm; 17-ml total volume) with a butyl rubber septum and subsequent streaking of the highest dilutions with visible growth on agar plates (2%) containing inulin (0.15%) or fructose (10 mM). These plates were incubated in a 2.5-liter jar at 58°C under an N_2-CO_2 atmosphere (80:20, vol/vol) to which 0.7 ml of a 10 mM Na_2S solution was added in order to maintain anaerobic conditions. Manipulation of agar plates and transfer to the jars was done in an anaerobic cabinet containing an N_2-CO_2-H_2 atmosphere (75:20:5, vol/vol/vol). After repeated plating, the strains were isolated in pure culture.

Growth experiments. Substrate tests and growth experiments at different pH values and temperatures were performed in culture tubes containing 9.5 ml of buffered basal medium to which 0.5 ml of a concentrated stock solution of the carbon and energy source was added (final concentrations depended on the substrate used, as indicated above). Immediately before inoculation, Na_2S was added to establish completely reduced conditions. The relationship between μ_{max} and pH was determined in MES (morpholineethanesulfonic acid)-buffered medium at low pH (5.5 to 7) and in Tris hydrochloride at high pH (7 to 9). All substrates used were filter sterilized (0.2-μm-pore-size filters) as concentrated stock solutions.

The enzyme and product formation during growth was studied with batch cultures grown in 600-ml bottles filled with 250 ml of medium to which the appropriate substrate had been added.

Chemical analyses. Alcohols and short-chain fatty acids were analyzed by gas chromatography (23, 29). Formate was measured by the colorimetric assay of Lang and Lang (24). H_2 and CO_2 in the gas phases of the cultures were analyzed with a Pye Unicam 104 gas chromatograph equipped with a thermal conductivity detector (23). Organic carbon was measured with a Shimadzu Total Organic Carbon Analyzer (model 500). The sugar content of inulin was determined after complete hydrolysis with 0.3 N HCl by the method of Somogyi (42).

Inulinase assay. By measuring the formation of reducing sugars by the method of Somogyi, inulinase activity in whole-cell cultures, in culture supernatants, and in suspensions of washed cells in 30 mM phosphate buffer was measured. The reaction mixture was composed of 2 ml of sodium phosphate buffer (30 mM, pH 6.8) containing inulin (6 g liter^{-1}) and 1.8 ml of sample. The cells in this reaction mixture were inactivated by flushing with air for 30 s. No formation of fermentation products was detected during the enzyme assays, indicating that cell metabolism had been blocked effectively. No difference in enzyme activity between completely anaerobic and air-flushed samples was detected, indicating that oxygen had no effect on the inulinase activity measured. One unit of inulinase activity is defined as the amount of enzyme liberating 1 μmol of reducing sugars per min. The amount of reducing sugars liberated by the inulinase(s) during the enzyme assay was linear both with time and with the amount of enzyme containing sample added. In order to compare the inulinase activity of our isolates with those of other inulin-degrading micoorganisms, the inulinase activity is expressed as units per gram of inulin degraded per liter.

Other methods. Absorbance of cell cultures in a 1-cm cuvette was measured at a wavelength of 660 nm with a Vitatron colorimeter. Cells taken from a batch culture of strain IC in the mid-log phase were used for Gram staining according to the method described previously (16). Cells of exponentially growing cultures of *Lactococcus lactis* and *Escherichia coli* served as controls. The Gram reaction was also examined by the KOH test of Gregersen (18). The presence of cytochromes was investigated by recording air-oxidized and dithionite-reduced spectra of whole cells and cell extracts with an Aminco type DW2a spectrophotometer. Cells were taken from an exponentially growing culture with an optical density at 660 nm of approximately 0.35 and concentrated 20-fold after centrifugation. The G+C moles percent was determined by the Deutsche Sammlung von Mikroorganismen (DSM) (Brunswick, Federal Republic of Germany) through buoyant density measurements after ultracentrifugation, with *Micrococcus luteus* DNA as a reference.

RESULTS

Isolated strains. Three succinate-forming strains were isolated in batch cultures, each from a different source: beet pulp from a sugar refinery, soil around a Jerusalem artichoke, and fresh cow manure. By using mud from a tropical pond of a botanical garden, a related strain was enriched in an inulin-limited continuous culture run at a low dilution rate (0.02 h^{-1}) for 22 days. From this chemostat enrichment, the predominant inulin-fermenting organism was isolated from the most diluted positive culture tube (10^{-8}) of the dilution series.

Characteristics of the isolated strains. The cells of the isolated thermophilic strains were rod shaped, 2 to 4 μm in length, and 0.3 to 0.4 μm in width (Fig. 1A and B). Electron microscopy revealed that the cells of the type strain, IC, are peritrichously flagellated (Fig. 1B). Spores were frequently observed in stationary-phase cultures (Fig. 1C). The diameter of the spores was approximately 0.6 μm. Thin sections of embedded cells showed the presence of a highly structured, multilayered cell surface (Fig. 1D). A surface view of a part of the S layer showed a regular, presumably hexagonically arranged surface structure (Fig. 1E).

The cells were negative in a Gram stain, but no lysis occurred during the KOH test, which is typical for a gram-positive bacterium.

Colonies on agar media were small, white, and smooth. They appeared within a few days of incubation in an anaerobic jar, on both nutrient broth and basal medium agar plates, with inulin as the growth substrate.

All four isolated strains were able to grow on fructose, glucose, galactose, xylose, ribose, sucrose, lactose, maltose, cellobiose, raffinose, and starch (monosaccharides, 10 mM each; disaccharides, 5 mM each; trisaccharides, 3.33 mM; and starch, 1.5 g/liter). No growth was observed with methanol (both 10 mM and 30 mM tested), glutamate, glycerol, pyruvate, citrate (30 mM each), arabinose (10 mM), Casamino Acids (5 g/liter), xylan, pectin, or cellulose (1.5 g

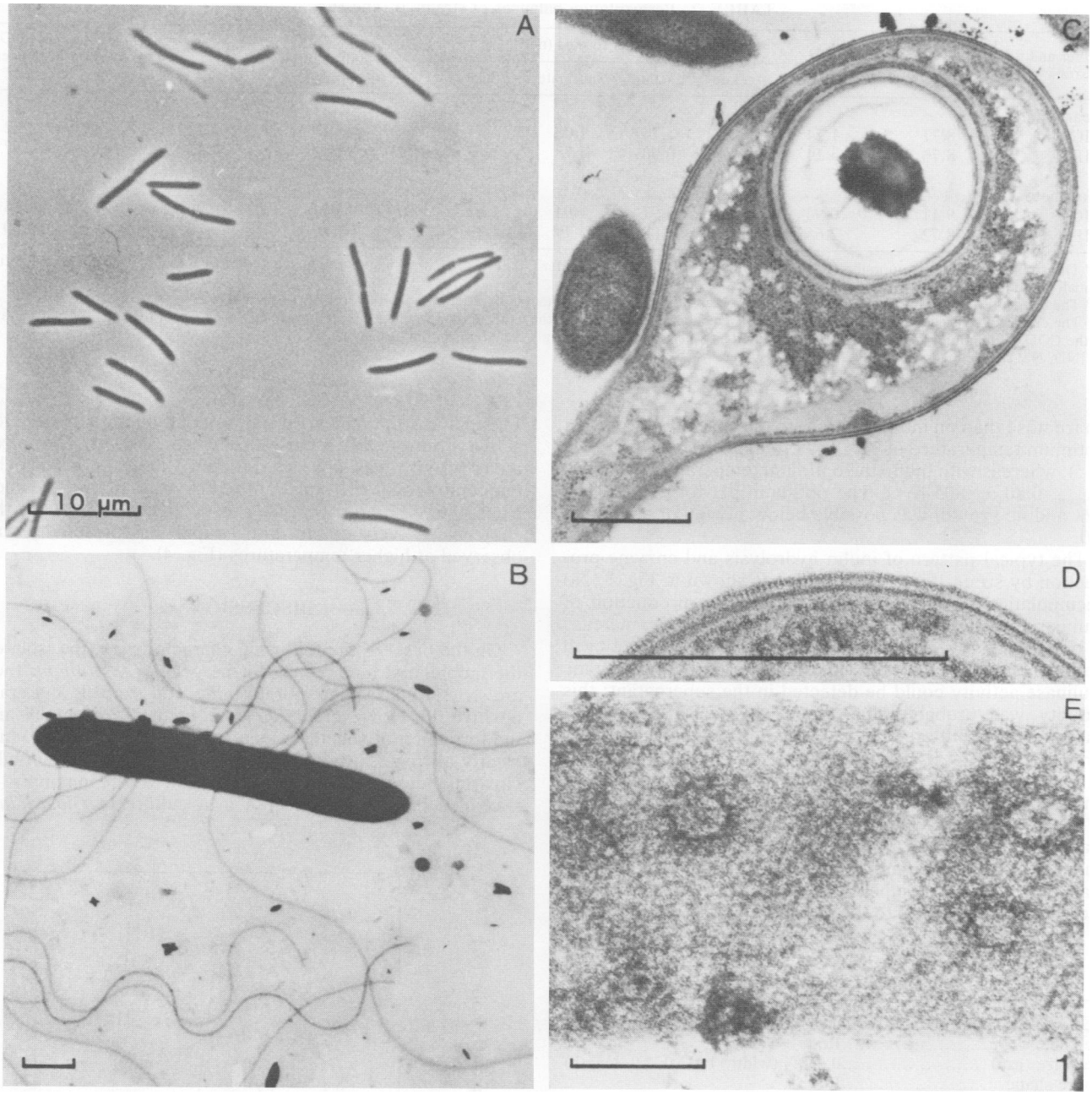

FIG. 1. (A) Phase-contrast photograph of vegetative cells harvested from an exponentially growing culture. (B) Electron micrograph of a peritrichously flagellated cell. (C) Ultrathin section of an embedded sporulating cell showing terminal swelling. (D) Regular surface layer as revealed in thin section of an embedded cell. (E) Negatively stained fragment of the surface layer indicating a regular arrangement of particles. Bars represent 0.5 μm unless stated otherwise.

of each per liter). Galactose, mannitol, sorbitol, and yeast extract were used by some but not all strains (Table 1). The products formed during growth on fructose, glucose, or inulin were succinate, formate, acetate, lactate, ethanol, and H_2. In Table 2, fermentation balances for two strains grown on inulin are presented. In Table 3, the major characteristics of strain IC are summarized.

Growth experiments. The relationship between μ_{max} and temperature depended on the growth substrate. Both the optimum and maximum temperature for growth were higher

TABLE 1. Substrates used only by some but not all of the isolated strains

Substrate	Use[a] by strain:			
	IA	IC	ID	IE
Galactose (10 mM)	−	+	+	+
Mannitol (10 mM)	±	−	−	±
Yeast extract (2 g/liter)	+	±	−	−
Sorbitol (10 mM)	−	−	−	+

[a] −, <15% of cell density obtained with 10 mM fructose; ±, ≤25% and ≥15% of cell density obtained with 10 mM fructose; +, >25% of cell density obtained with 10 mM fructose.

TABLE 2. Fermentation balances of strains IC and IE[a]

Buffer and strain	Inulin fermented (mM reducing sugars)[b]	Concn of product formed (mM)						CO_2 consumption (mM)	Cell material (mM carbon)	Carbon recovery (%)	COD recovery (%)[c]
		Formate	Acetate	Lactate	Succinate	Ethanol	H_2				
Bicarbonate											
IC	8.71	1.24	11.96	1.74	2.08	1.44	0.6	ND[d]	9.22	ND	102
IE	8.76	3.14	6.54	0.00	5.78	1.63	0.51	ND	9.41	ND	97
Phosphate											
IC	9.11	0.94	13.98	0.91	1.94	1.42	1.09	1.60	10.90	94	103
IE	8.72	2.65	6.82	0.00	5.79	1.99	0.95	3.01	9.36	95	100

[a] The strains were grown on inulin (1.5 g liter^{-1}) in either an $NaHCO_3$ buffer (30 mM, pH 6.8) or a sodium phosphate buffer (30 mM, pH 6.8). To the latter a small amount of $NaHCO_3$ was added (5 mM).
[b] The concentration of sugars in inulin was measured by the method of Somogyi (42) after complete hydrolysis of the polymer with 0.3 N HCl.
[c] The chemical oxygen demand (COD) recovery is calculated as the ratio of the COD of the fermentation products plus the CODs of the cells formed relative to the COD of the inulin fermented and is expressed as a percentage (assuming a cell composition of $CH_{1.8}O_{0.5}$).
[d] ND, Not determined.

on fructose than on inulin (Fig. 2). In the case of fructose, an optimum temperature of 72°C was found (μ_{max}, 0.40 ± 0.05 h^{-1}), whereas with inulin the optimum temperature was 58°C (μ_{max}, 0.80 ± 0.05 h^{-1}). The optimum pH for growth was 7.6, and no growth was possible below pH 6.0 or above pH 9.0.

The typical pattern of inulin hydrolysis and enzyme production by strain IC in batch culture is shown in Fig. 3. No accumulation of sugars was observed. The production of inulinase in batch culture proceeded parallel to the increase in optical density. The enzyme was almost exclusively cell bound (Fig. 3). However, in the stationary phase some inulinase activity could be detected in the supernatant; this activity was probably due to cell lysis. During growth experiments in buffered batch cultures, no significant drop in pH was observed (<0.4 pH unit).

Some properties of the inulinase activity. In an inulin-limited anaerobic chemostat culture at a dilution rate of 0.05 h^{-1}, the total (cell-bound plus cell-free) inulinase activity was comparable with that in batch cultures. However, in chemostat cultures approximately 30% of the total activity was present in the supernatant, probably as a result of increased cell lysis due to the low dilution rate (6% of μ_{max}).

TABLE 3. Major characteristics of strain IC[a]

Parameter	Characteristic
Cell shape	Rod
Cell size (μm)	0.3–0.4 (width) by 2.0–4.0 (length)
Spore shape	Round
Spore position	Terminal
Motility	+
Gelatin hydrolysis	Weak (2 wk)
Esculin hydrolysis	+
Cellulose degradation	−
Gas production	+
Thiosulfate reduction to:	
Sulfide	+
Sulfur	−
Nitrate reduction	−
Peptidoglycan type of the cell wall	LL-Diaminopimelic acid
Milk reaction	Curd, acid, gas
Gram staining	−
KOH test	+

[a] For discussion of substrate spectrum, colony formation, and ultrastructure, see text. The tests not described in Materials and Methods were performed by the DSM according to standard methods.

The temperature and pH profiles of cell-bound and cell-free activity were nearly identical (data not shown). The maximum activity was observed at 58°C and at pH 6.8. The temperature and pH range of the enzyme activity stretched from 30 to 72°C and 5.8 to 8.4, respectively. Up to 60°C the enzyme was very stable, but a rapid loss of activity was observed at higher temperatures (Fig. 4).

DISCUSSION

On the basis of the following characteristics, the isolated thermophilic strains belong to the genus *Clostridium*: they are strictly anaerobic, sporeforming, rod shaped, and gram positive (8). However, the G+C content of 35.9 mol% and the formation of succinate as a major fermentation product clearly distinguish these isolates from all other thermophilic clostridia described so far (Table 4). Trace amounts of succinate have been detected in a culture of *Clostridium*

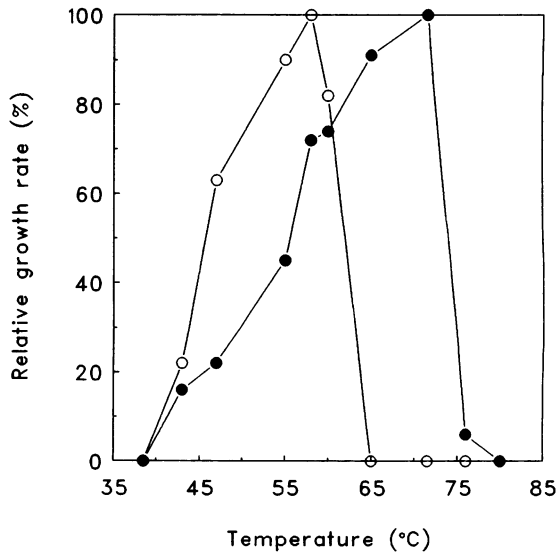

FIG. 2. Effect of temperature on the specific growth rate of strain IC, with inulin (1.5 g liter^{-1}) or fructose (10 mM) as the carbon and energy source. The specific growth rate is expressed as a percentage of the maximum specific growth rate at the optimal temperature (0.80 and 0.4 h^{-1}, respectively). Symbols: ●, μ_{max} with fructose; ○, μ_{max} with inulin.

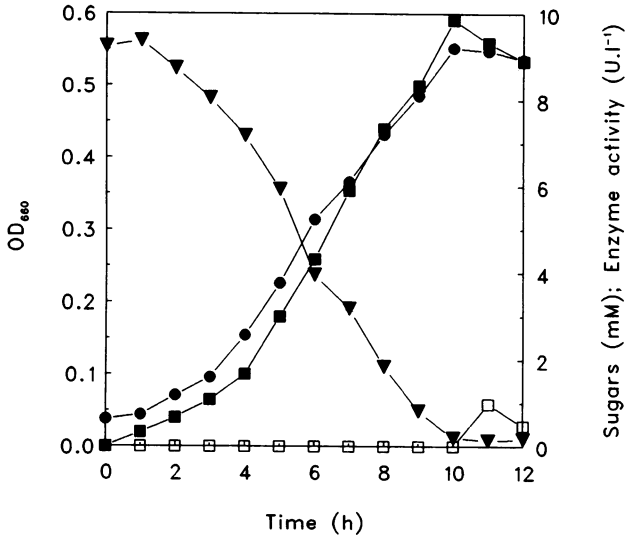

FIG. 3. Kinetics of growth and inulinase activity of strain IC in batch culture on inulin-containing medium (1.5 g of inulin liter^{-1}). Symbols: ●, optical density at 660 nm (OD_{660}); ■, cell-bound inulinase activity; □, cell-free inulinase activity; ▼, concentration of inulin, expressed as millimolar fructose concentration.

thermosaccharolyticum (8), and in a culture of *Clostridium thermocellum* grown on sugar beet pulp under ill-defined conditions, some succinate has been detected as a fermentation product (32). Thus, it appears that these new isolates represent the first example of a thermophilic clostridium forming significant amounts of succinate during fermentation on sugars in defined mineral medium. In the case of the mesophilic clostridia, a limited number of species produced moderate amounts of succinate when grown on PYG broth (8). Only in the case of *Clostridium coccoides* was succinate detected as a major fermentation product (8). In addition to succinate, our isolates formed other fermentation products, such as formate, H_2, acetate, ethanol, and sometimes lactate. On the basis of the complete fermentation balance presented in Table 2, the following overall reactions are proposed for the fermentation of inulin in phosphate-buffered medium by strains IC and IE, with inulin expressed as hexose units (10, 47):

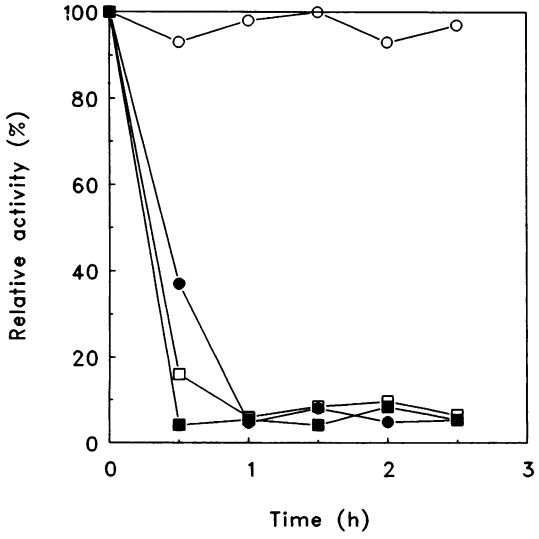

FIG. 4. Effect of temperature on stability of inulinase in cell cultures, expressed as a percentage of the initial total activity (13.76 U liter^{-1}). Symbols: ○, 58°C; ●, 63°C; □, 67°C; ■, 70°C.

100 hexose + 21.9 CO_2 → 12.9 formate + 192.4 acetate + 12.5 lactate + 26.6 succinate + 19.5 ethanol + 15.0 H_2 (strain IC)

$\Delta G'$ (25°C) = −51.1 kJ mol^{-1}; $\Delta G'$ (58°C) = −52.6 kJ mol^{-1}

100 hexose + 42.1 CO_2 → 37.1 formate + 95.4 acetate + 80.5 succinate + 27.8 ethanol + 13.3 H_2 (strain IE)

$\Delta G'$ (25°C) = −52.5 kJ mol^{-1}; $\Delta G'$ (58°C) = −15.9 kJ mol^{-1}

This pattern of product formation closely resembles a mixed-acid type of fermentation characteristic of many facultatively aerobic bacteria, including several members of the family *Enterobacteriaceae* and many *Bacillus* species (4, 9). Furthermore, mesophilic clostridia with a similar pattern of fermentation have been described (8). An important characteristic of this fermentation is its variability with respect to the precise quantities of the various products (31, 44). The extent to which this also applies to our thermophilic strains is not yet entirely clear, but preliminary results indicate that specific growth rate, temperature, pH, and H_2 and CO_2 availability do affect the fermentation balances (further studies are in progress). However, it is evident from the present results that considerable differences between strains of these clostridia exist. Especially noteworthy in this respect is the different relationship between the temperature and the $\Delta G'$ for product formation by different strains (compare the fermentation balances of strains IC and IE [Table 2]). This result may indicate significant differences in the way in which the fermentative metabolism in these two strains is regulated in response to temperature changes. The extent to which such temperature changes indeed affect the fermentation patterns in a different manner requires further experimentation.

An interesting ecological aspect of the isolated clostridia is their occurrence in habitats which probably never reach "thermophilic" temperatures (50 to 60°C [45, 46]). Only one of the four strains described above was obtained from soil artificially kept at a temperature of around 50°C. In an elegant study, Wiegel et al. (52) demonstrated that thermophiles are very widespread. These researchers isolated *Clostridium thermohydrosulfuricum* from 13 different places, including soils with moderate temperatures, a sewage plant, and hot springs. However, the question of whether these thermophilic microorganisms are really ever active in the "mesophilic" habitats with ambient temperatures far below 50 to 60°C remains unanswered. Studies of the distribution of thermophiles along the temperature gradient of hot springs revealed that the optimal temperature of many microorganisms was indeed similar to the environmental temperature (5, 6, 55). On the other hand, Sandbeck and Ward (39) showed that thermophilic methanogens isolated at 50 and 55°C were not adapted to the environmental temperature but grew optimally at 65°C. Similar results were found by Benner and Vaun-McArthur (3) in a study of the effects of temperature on microbial utilization of lignocellulosic detritus in the Savannah River in South Carolina. Whereas the ambient temperature ranged from 5 to 35°C during the spring and always remained below 20°C during the winter, the optimum

TABLE 4. Major differences between thermophilic *Clostridium* species

Species	Growth on cellulose	Growth on H_2-CO_2	Fermentation product(s)[a]	G+C mol%	Reference
C. thermocellum	+	−	A, E, B, l	38–39	14
C. thermocopriae	+	−	B, A, L, E	36.7–37.8	8
C. stercorarium	+	−	A, E, l	39	8
C. fervidus	−	−	A	39	34
C. thermoaceticum	−	+	A	54	50
C. thermoautotrophicum	−	+	A	53–55	8
C. thermosaccharolyticum	−	−	B, a, l	29–32	8
C. thermosulfurogenes	−	−	E, L, A	32.6	40
C. thermohydrosulfuricum	−	−	E, l, A	29.5–32	52
C. thermolacticum	±	−	L, a, E	40.9–42.3	38
C. thermobutyricum	−	−	B, l, a	37	51
C. thermosuccinogenes	−	−	A, L, S, E	35.9 ± 1	This paper

[a] A, Acetate; B, butyrate; E, ethanol; L, lactate; S, succinate. Capital letters are used for fermentation products formed in significant amounts, whereas lowercase letters indicate minor amounts.

temperature for lignocellulose degradation never decreased below 35°C. In order to obtain an understanding of the possible role of thermophilic bacteria living in soils with moderate temperatures, experiments addressing this question directly are badly needed. First of all, it is necessary to learn more about the relative distribution of both aerobic and anaerobic thermophilic bacteria in different habitats. Furthermore, it is necessary to determine whether in soils these thermophiles are really metabolically active or predominantly present in the spore form. Since growth of these thermophiles in soils occurs at suboptimal temperatures, studies of their microbial physiology at such temperatures are urgently needed. With respect to the thermophilic inulin-fermenting clostridia, this type of study has recently been started in our laboratory.

Research on thermophilic, anaerobic microorganisms has proven very fruitful for the use of such organisms in biotechnological processes (43, 54). The use of inulinases from thermophilic microorganisms is very promising because elevated temperatures ensure high solubility of inulin, a major limiting factor in obtaining high conversion rates at low temperatures (48). The temperature optimum of 58°C and the thermostability at up to 60°C for the inulinases produced by *C. thermosuccinogenes* are among the highest reported thus far (49). The enzymes of strain IC are more thermostable than those of the biotechnologically important yeast *Kluyveromyces marxianus* (37) and are comparable to the inulinases which have been isolated from *Aspergillus niger* (48). Unfortunately, the total activity of the inulinases present at the end of growth in a batch culture of strain IC is very low (6.9 U g of inulin degraded^{-1} liter^{-1}) compared with that of yeasts (21,200 U g of inulin degraded^{-1} liter^{-1} [33]). However, it is on the same order of magnitude reported for *Clostridium acetobutylicum* (21.8 U g of inulin degraded^{-1} liter^{-1} [26]).

Species description. On the basis of the G+C content of strain IC (35.9 mol%) and the formation of succinic acid as a major fermentation product by all of the newly isolated thermophilic, inulin-hydrolyzing strains, we propose to create a new species within the genus *Clostridium*, namely *Clostridium thermosuccinogenes* (ther.mo'suc.ci.no'ge.nes. Gr. adj. *thermos*, hot; M.L. n. *acidum succinicum*, succinic acid; Gr. v. *gennaio*, produce; M.L. adj. *thermosuccinogenes*, succinic acid producing in heat).

(i) Cellular characteristics. Straight rods 2 to 4 μm in length and 0.3 to 0.4 μm in width; peritrichously inserted flagella are present. Surface layer consists of hexagonally arranged particles; terminal spores encountered frequently towards the end of the growth phase in batch culture; cells stained negative in Gram stain.

(ii) Colony characteristics. Small (1 to 2 mm in diameter), white, round colonies are formed within a few days of incubation in an anaerobic jar, on both nutrient broth and basal medium agar plates supplied with inulin.

(iii) Physiological characteristics. Strictly anaerobic growth was possible on a range of sugars. These include fructose, glucose, galactose, inulin, xylose, ribose, sucrose, lactose, maltose, cellobiose, raffinose, and starch. No growth was observed with methanol, glutamate, glycerol, pyruvate, citrate, arabinose, Casamino Acids, xylan, pectin, cellulose, or H_2-CO_2 (80:20, vol/vol). Some strains fermented galactose, mannitol, yeast extract, and sorbitol. Formate, acetate, lactate, succinate, and H_2 were the fermentation products when inulin was used as the growth substrate.

The G+C content of the type strain, IC, is 35.9 mol%.

(iv) Habitats. Apparently widespread, as the organism could be enriched from different sources, including fresh cow manure, beet pulp from a sugar refinery, soil around a Jerusalem artichoke, and mud from a tropical pond of a botanical garden.

Strain availability. The type strain, IC, has been deposited as DSM 5807^T in the DSM. The other strains, IA, ID, and IE, have also been deposited in the DSM as DSM 5806 (strain IA), DSM 5808 (strain ID), and DSM 5809 (strain IE).

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