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Characterization of a Symbiotic Coculture of *Clostridium thermohydrosulfuricum* YM3 and *Clostridium thermocellum* YM4

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***Clostridium thermohydrosulfuricum* YM3 and *C. thermocellum* YM4 were isolated from a coculture which was obtained from an enrichment culture inoculated with volcanic soil in Izu Peninsula, Japan. Strain YM3 had advantages over reported *C. thermohydrosulfuricum* strains in that it fermented inulin and could accumulate ethanol up to 1.3% (wt/vol). The highest ethanol yield obtained was 1.96 mol/mol of anhydroglucose unit in cellobiose. Strain YM4 had features different from those reported in *C. thermocellum* strains: it formed spores rarely (at a frequency of $<10^{-5}$), it required CO_2 and Na_2CO_3 for growth, and it fermented sucrose. Strain YM4 completely decomposed 1% Avicel within 25 h when the inoculum constituted 2% of the culture medium volume, and it produced 0.22 U of Avicelase and 2.21 U of carboxymethylcellulase per ml of the medium. The doubling times on Avicel, cellobiose, and glucose were 2.7, 1.1, and 1.6 h, respectively. Reconstructed cocultures of strains YM3 and YM4 were very stable and degraded Avicel more rapidly than did strain YM4 monoculture. Without yeast extract, neither microorganism was able to grow. However, the coculture grew on cellulose without yeast extract and produced ethanol in high yield. Moreover, cell-free spent culture broth of strain YM3 could replace yeast extract in supporting the growth of strain YM4. The symbiotic relationship of the two bacteria in cellulose fermentation is probably a case of mutualism.**

Cellulose is the most abundant biopolymer on Earth and makes up a major component of municipal, agricultural, and industrial wastes. Attempts at microbial conversion of cellulosic substances into fuels and chemicals have been made. Among these, the production of ethanol by using a thermophilic anaerobic bacterium, *Clostridium thermocellum*, has been intensively studied (4, 15, 24) and has attracted wide attention for its ability to convert substrates directly to ethanol. Cocultures of *C. thermocellum* with saccharolytic ethanologenic bacteria such as *C. thermosaccharolyticum* (20), *C. thermohydrosulfuricum* (14), and *Thermoanaerobacter ethanolicus* (L. G. Ljungdahl and J. Wiegel, U.S. patent 4,292,406, Sept. 1981) have been reported to be very effective in enhancing ethanol yield. A naturally occurring coculture of the mesophiles, *Bacteroides cellulosolvens* and *C. saccharolyticum*, has also been reported (12).

In a previous paper, we reported the isolation of a coculture of thermophilic anaerobes which was capable of degrading 2% cellulose, xylan, and starch within 72 h (with a 2% inoculum) and produced about 8 g of ethanol per liter (11). Although it had gone through single-colony isolation procedures more than 10 times with roll tube agar medium containing either cellulose, cellobiose, or glucose as an energy source, this coculture seemed to be composed of at least two kinds of bacteria. This was indicated by the different appearances of colonies in xylose medium from those in cellulose or cellobiose medium, their absence of cellulolytic activity, and their production of more ethanol from cellobiose and glucose than did the original culture. The purposes of this study were to purify the bacteria in the coculture, characterize them, and find reasons for the stability of the association.

MATERIALS AND METHODS

Microorganisms and culture conditions. *C. thermohydrosulfuricum* YM3 was purified from the coculture designated as IB22, which was obtained from soil collected in the volcanic area on Izu Peninsula, Japan (11), by picking colonies in a xylose-agar shake roll tube culture incubated at 60°C or in a glucose-agar shake roll tube culture at 70°C. After attempts to purify *C. thermocellum* YM4 by single-colony isolation and micromanipulation techniques failed, it was purified by the procedure described below. Coculture at the mid-logarithmic phase in the cellobiose-liquid medium was diluted 10^7-fold with sterile distilled water. A series was made of one drop of suspension placed on sterile small agar chips (8 by 8 mm). The chips were observed under a dark-field microscope at magnifications of ×40 to ×400. Chips on which only one bacterial cell was found were cultivated in a cellulose-agar shake roll tube medium at 60°C. The resulting colonies lacked the ability to grow on xylose at 60°C or on glucose at 70°C, which ensured the purity of *C. thermocellum*.

Strains YM3 and YM4 were grown, as described earlier (8), under an atmosphere of CO_2 in a medium containing (per liter) 0.45 g of K_2HPO_4, 0.45 g of KH_2PO_4, 0.9 g of $(NH_4)_2SO_4$, 0.9 g of NaCl, 0.18 g of $MgSO_4 \cdot 7H_2O$, 0.12 g of $CaCl_2 \cdot 2H_2O$, 5 g of yeast extract, 1 ml of 0.1% hemin, 1 ml of 0.1% resazurin, 2 ml of vitamin solution, 10 ml of trace mineral solution, and 10 or 20 g of carbohydrate (Avicel, cellobiose, etc.). The vitamin solution contained the following (in milligrams per liter) inositol, 1,000; calcium pantothenate, 200; niacin, 200; pyridoxine hydrochloride, 200; thiamine hydrochloride, 200; *p*-aminobenzoate, 100; riboflavin, 100; cyanocobalamin, 10; biotin, 5; and folic acid, 5. The trace mineral solution contained the following (in milligrams per liter): $FeSO_4 \cdot 7H_2O$, 150; H_3BO_3, 100; $MnSO_4 \cdot H_2O$, 80; $ZnSO_4 \cdot 7H_2O$, 80; $Na_2MoO_4 \cdot 2H_2O$, 40; $Na_2WO_4 \cdot 2H_2O$, 40; KI, 20; $NiCl_2$, 10; $CoCl_2$, 10; Na_2SeO_3, 3; $CuSO_4 \cdot 5H_2O$, 4; and $AsCl_3$, 3. The pH of the medium was adjusted to 7.2 with 2 N NaOH, and it was boiled and then bubbled with CO_2. Then 50 ml of 8% Na_2CO_3 and 30 ml of freshly prepared 1% cysteine hydrochloride were added. The medium was dispensed in 50- or 80-ml portions into 125-ml vials, which were sealed with butyl rubber stoppers and autoclaved. The pH of the medium equilibrated with

CO_2 was about 6.8. When medium was prepared for a jar fermentor, the Na_2CO_3 and cysteine hydrochloride solutions were autoclaved separately and then added. *C. thermohydrosulfuricum* YM3 was grown with shaking (100 strokes per min; amplitude, 4 cm) in the serum vials, usually at 66°C, with cellobiose as the carbon source. *C. thermocellum* YM4 and the coculture with strain YM3 were incubated at 60°C in the serum vials with shaking or in a 3-liter jar fermentor (Iwashiya Co., Tokyo, Japan) containing 2 liters of the medium with agitation at 60 rpm, without pH adjustment. Avicel (type SF; Asahikasei Co., Tokyo, Japan) was used as the carbon source.

Measurements of growth and residual cellulose. Cultures that contained cellulose were centrifuged at 50 × *g* for 10 min, and the optical density at 660 nm of the supernatant was measured as a growth parameter. The pellet was suspended in 8% formic acid to lyse the cells and centrifuged at 50 × *g* for 10 min. After one more wash with and resuspension in 8% formic acid, the solution was passed through preweighed membrane filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The filters were dried at 60°C to constant weight, and the amount of residual cellulose was determined by difference.

DNA composition analysis. DNA from about 5 g (wet weight) of cells was isolated by the procedure of Saito and Miura (16). The percent guanosine-plus-cytosine content of DNA was determined by thermal denaturation (9) with a recording spectrophotometer (no. 2500S; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) fitted with a thermal programmer.

Cellulase determination. Culture broth was mixed with 3 volumes of MES [2-(*N*-Morpholino)ethanesulfonic acid]-NaOH buffer (0.05 M, pH 6.0) and centrifuged at 16,000 × *g* for 10 min. The supernatant was used for the cellulase assay. Avicelase activity was measured by incubating 4 ml of the enzyme solution with 40 mg of Avicel at 65°C for 1 h with constant shaking. The amount of reducing sugars liberated was determined by the colorimetric method of Nelson (13) as improved by Somogyi (18). The carboxymethylcellulase (CMCase) activity in the MES buffer was assayed by the method of Miller et al. (10) with CMC sodium salt (Sigma Chemical Co., St. Louis, Mo.) as the substrate. One unit of enzyme releases 1 μmol of reducing sugar per min.

Product analysis. Organic acids and alcohols were determined by using a gas chromatograph (Shimazu GC-7AG) equipped with a flame ionization detector. A glass column (3 mm by 2 m) packed with Gasukuropack 54 (Gasukurokogyo Co., Tokyo, Japan) was used. The chromatogram was run at 170°C with N_2 as the carrier gas at a flow rate of 40 ml/min. Lactic acid was assayed by using lactic dehydrogenase (Boehringer GmbH, Mannheim, Federal Republic of Germany). Ion-exchange chromatography with a model S-700 carboxylic acid analyzer (Seishin Pharmaceutical Co., Tokyo, Japan) was also used for organic acid analysis. H_2 and CO_2 were determined by using a gas chromatograph (Shimazu GC-4B) with a thermal conductivity detector and a 2-m glass column containing active carbon. The column temperature was 100°C, with N_2 as the carrier gas at a flow rate of 40 ml/min. For determination of CO_2 solubilized in the culture medium, a 0.5-ml sample was transferred to a 4.5-ml serum vial sealed with a butyl rubber stopper and containing 0.5 ml of concentrated sulfuric acid. The liberated CO_2 was analyzed as described above after the vial had been shaken and its temperature had dropped to room temperature. Reducing sugars were determined by the phenol-H_2SO_4 method of Dubois et al. (3).

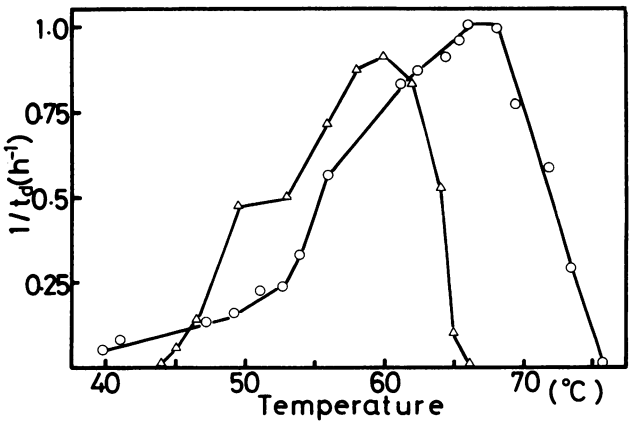

FIG. 1. Effect of temperature on the growth rates ($1/t_d$; t_d = doubling time) of *C. thermohydrosulfuricum* YM3 (○) and *C. thermocellum* YM4 (△). Strains YM3 and YM4 were grown with shaking in serum vials containing media with 1% glucose and cellobiose, respectively, as carbon sources.

Test conditions for a symbiotic relationship. Cultures for tests on symbiosis were grown in serum bottles containing 80 ml of medium lacking the vitamin solution. Strain YM3 cell-free spent culture broth was prepared from a 4-day-old culture on 1% glucose–0.05% yeast extract by being passed through a 0.22-μm membrane filter (Millipore). Reducing sugars were not detectable in that broth. Growth on cellulose with various amounts of yeast extract was compared for the coculture and for strain YM4 monocultures with or without strain YM3 broth. Monocultures were initiated by inoculation of 2 ml of strain YM4 cultures with 16 ml of the strain YM3 cell-free broth or sugar-free medium that was identical to the one used for preparing the cell-free broth except that it lacked glucose. The medium used for monocultures contained 1.2% Avicel to give a final concentration of 1.0% (inocula not considered). Cocultures were initiated by the simultaneous inoculation of 2-ml cultures of each microorganism. All cultures were incubated at 60°C with shaking.

RESULTS

Properties of *C. thermohydrosulfuricum* YM3. Strain YM3 was isolated as described in Materials and Methods. It was an obligately anaerobic, gram-negative rod (0.4 to 0.6 by 1.5 to 4 μm), which formed terminal oval spores, was peritrichous, and was sluggishly motile. The DNA content was 31.5 mol% guanosine plus cytosine. Figure 1 shows the relationship between the doubling time and the growth temperature. The optimum temperature was 66 to 68°C, and no growth was observed at temperatures below 35°C or above 76°C. The pH optimum was between 6.8 and 7.1, but growth occurred between pH 5.5 and 9.0 (Fig. 2). H_2S was formed from sulfite. Acetyl methyl carbinol was not produced. Nitrate was not reduced. Gelatin was not liquefied, and indole was not produced. Strain YM3 did not ferment cellulose but did ferment the following substrates to produce ethanol, acetate, lactate, formate, H_2 and CO_2: D-glucose, D-fructose, D-mannose, D-galactose, D-xylose, D-ribose, cellobiose, sucrose, maltose, lactose, trehalose, melibiose, raffinose, starch, xylan, pectin, inulin, sorbitol, mannitol, dulcitol, salicin, and glucuronic acid. Electron microscopic examinations by using the freeze-etching technique clearly showed that the outer layer of the cell wall of strain YM3 had hexagonally arranged particles. The doubling times at 66°C

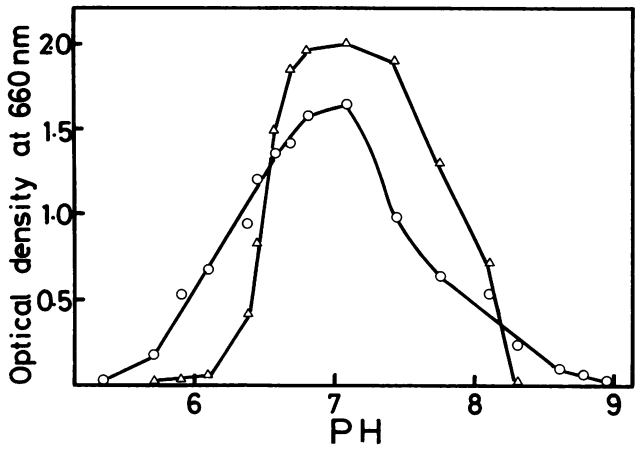

FIG. 2. Effect of initial medium pH on the growth of *C. thermohydrosulfuricum* YM3 (○) and *C. thermocellum* YM4 (△). Strains YM3 and YM4 were grown as indicated in the legend to Fig. 1, but at 60°C and at different pH values. The pH of the medium was adjusted by the addition of 7 N NaOH or 99.5% acetate, which were not significantly inhibitory to the growth of the strains at the concentrations used. Precultures grown at pH 6.8 were used as inocula. The optical density at 660 nm was measured after a 12-h incubation.

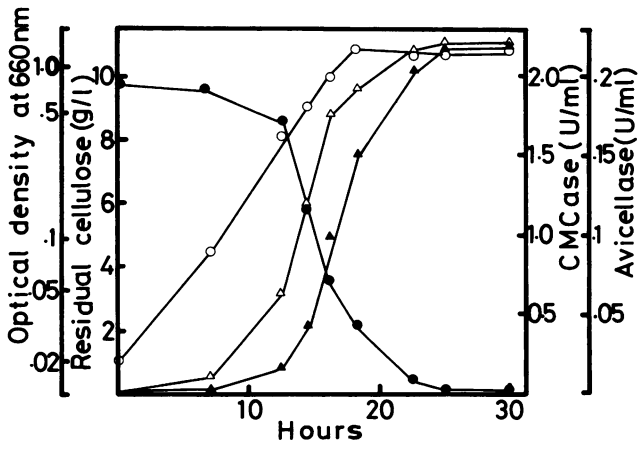

FIG. 3. Degradation of cellulose and production of cellulases by *C. thermocellum* YM4. The cultivation was carried out at 60°C in a 3-liter jar fermentor under CO_2 with agitation at 60 rpm. The medium contained 1% Avicel. The pH was not controlled. Five 5-ml samples were withdrawn each time for determination of residual cellulose (●), optical density at 660 nm (○), CMCase activity (△), and Avicelase activity (▲).

were 1.0 h on glucose and 1.3 h on cellobiose. Strain YM3 produced large quantities of ethanol from various saccharides. The highest ethanol yield (1.96 mol/mol of anhydroglucose unit) was attained when 2% of cellobiose (117 mM glucose equivalents) was used as the substrate (Table 1). The highest ethanol concentration accumulated was 1.3% (wt/vol).

Properties of *C. thermocellum* YM4. Strain YM4 was an obligately anaerobic, gram-negative rod (0.4 to 0.6 by 2 to 4 μm). Spores were rarely formed (frequency, $<10^{-5}$) when it was grown on cellulose. No spores were found in the cultures of cellobiose-agar or cellobiose-liquid medium, regardless of the time of harvesting. When grown on cellulose or cellobiose, strain YM4 produced a yellow pigment. Microscopic examinations showed that many cells adhered to cellulose fibers. Strain YM4 fermented cellulose, cellobiose, salicin, glucose, fructose, sucrose, and sorbitol. The primary fermentation products were ethanol, acetate, lactate, H_2, and CO_2 (Table 1). The DNA base composition was 40.1 mol% guanosine plus cytosine. The temperature range for growth was 45 to 65°C, with an optimum around 60°C (Fig. 1). The pH optimum was between 6.8 and 7.1, but growth occurred between pH 5.9 and 8.1 (Fig. 2). In batch cultures, the pH could drop to 5.8. No growth was observed in medium lacking Na_2CO_3 with N_2 as the gas phase in place of CO_2, even when the pH had been adjusted to 6.8. The optimum Na_2CO_3 concentration for growth under an atmosphere of CO_2 was 0.4 to 0.6%. No or little growth was observed with an Na_2CO_3 concentration of 0.2% or lower, but it was not clear whether growth depends on Na_2CO_3, because the pH of the medium dropped out of the favorable range. A decrease in the growth rate was observed with an Na_2CO_3 concentration of 0.8% or more, although the pH of the medium was within the optimum range. Strain YM4 completely degraded 1% Avicel within 25 h when inoculated at 2% (vol/vol), and it produced 2.21 U of CMCase and 0.22 U of Avicelase per ml of the medium (Fig. 3). It also produced 0.12 U of Avicelase and 1.71 U of CMCase per ml when grown on cellobiose. The solubilization rate of Avicel during the most rapid period was about 1.1 g/liter per h. The doubling times on Avicel, cellobiose, and glucose were 2.7, 1.1, and 1.6 h, respectively.

Reconstruction of the coculture of strains YM3 and YM4. Strains YM3 and YM4 formed a stable coculture and produced a high yield of ethanol, as did the original coculture. Figure 4 illustrates a time course of Avicel fermentation by the coculture started with the same inoculum size as were the cultures of the separate microorganisms. Compared with the strain YM4 monoculture, decomposition of Avicel was more rapid, and increased ethanol (2.3-fold) and decreased

TABLE 1. Fermentation products of *C. thermohydrosulfuricum* YM3 and *C. thermocellum* YM4[a]

Strain	Substrate	Amt of following product formed (μmol/ml):						% Carbon recovery	O/R balance
		Ethanol	Lactate	Acetate	CO_2	H_2	Reducing sugars[b]		
YM3	1% Cellobiose	102	7.7	5.1	101	9.4	ND[c]	96.4	0.95
YM3	2% Cellobiose	229	10.0	7.8	231	13.1	ND	105	0.98
YM4	1% Avicel[d]	44.8	20.5	26.6	68.2	59.2	9.4	87.7	0.92

[a] Fermentations of strains YM3 and YM4 were carried out at 66 and 60°C, respectively, in serum vials containing 50 ml of the media. Cultures with 1 and 2% substrate were analyzed after 2 and 4 days of incubation, respectively. OR, Oxidation/reduction.
[b] Expressed as glucose equivalent.
[c] ND, Not detectable.
[d] 99% of the added Avicel (9.9 g/liter) was solubilized.

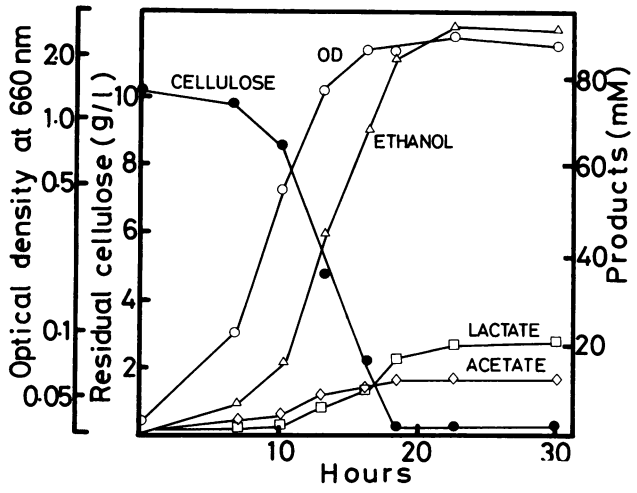

FIG. 4. Fermentation profile of coculture of *C. thermohydrosulfuricum* YM3 and *C. thermocellum* YM4 on 1% Avicel. Strains YM3 and YM4 were grown as indicated in the legend to Fig. 3.

acetate (0.6-fold) yields were observed. Reducing sugars in the spent medium was not detected. Ratios of the inoculum sizes of strains YM3 to YM4 from 1:5 to 5:1 with the total inoculum volume fixed had no significant effect on cellulose fermentation by the coculture at 60°C.

Symbiotic relationship between strains YM3 and YM4. Since the differences in the degradation rates of the monoculture of strain YM4 and the coculture were minor in the presence of sufficient yeast extract, nutritionally unfavorable conditions were used to study the interaction of the two strains in the coculture. In the absence of added vitamins, both strains required yeast extract for growth; the minimum amounts required were 0.05% for strain YM3 and 0.1% for strain YM4.

Cellulose degradation and growth at different yeast extract concentrations were compared for strain YM4 in monoculture, in monoculture with cell-free spent culture broth of strain YM3, and in the coculture (Fig. 5). For inocula, the fourth subcultures of strains YM3 and YM4 separately grown in the medium containing yeast extract at 0.05 and 0.1%, respectively, were used. Coculture with strain YM3 or addition of strain YM3 cell-free culture broth enabled strain YM4 to decompose cellulose and multiply even in the medium lacking yeast extract. At a yeast extract concentration of 0.1%, strain YM4 grew very slowly, but its growth and cellulose decomposition were greatly facilitated by coculture with strain YM3 or by addition of YM3 culture broth. In the medium supplemented with the same amount of yeast extract, the coculture produced a higher final optical density and CMCase activity than did the monocultures (Table 2). None or very little reducing sugar was detected in the final culture broths of the cocultures, whereas significant amounts remained in the monocultures of strain YM4, regardless of whether spent culture broth of strain YM3 was added. The end product ratios of ethanol to acetate were 4.3 to 10.5 for the cocultures and 1.8 to 1.9 for the monocultures, regardless of addition of the spent culture broth. This result confirms that strain YM3 actually grew in the coculture system, even in the medium not containing the minimal amount of yeast extract required for growth of strain YM3.

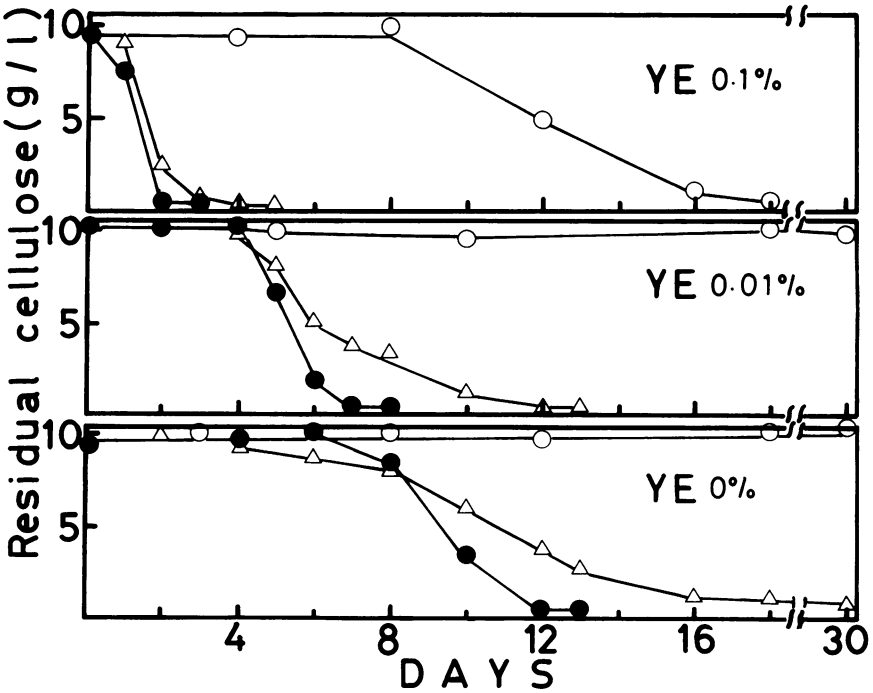

FIG. 5. Cellulose degradation by a *C. thermocellum* YM4 monoculture with or without the cell-free spent broth of *C. thermohydrosulfuricum* YM3 and by the coculture. The fourth subcultures of strains YM3 and YM4 in the medium containing yeast extract (YE) at 0.05 and 0.1%, respectively, were used for inocula. Cultivations were carried out at 60°C in serum vials containing 80 ml of the medium with 1% Avicel and yeast extract at 0, 0.01, or 0.1%. The vitamin solution was not added to the media used in this experiment. Symbols: ○, strain YM4 monoculture; △, strain YM4 monoculture with strain YM3 cell-free broth; ●, coculture.

TABLE 2. Comparison of fermentations of cellulose by *C. thermocellum* YM4 monoculture with and without *C. thermohydrosulfuricum* YM3 cell-free broth and by the coculture[a]

Yeast extract (%)	Conditions	Final OD[b]	CMCase (U/ml)	Amt of following product formed (mM):			Amt of reducing sugars (g/liter)	Amt of cellulose solubilized (g/liter)
				Ethanol	Lactate	Acetate		
0.1	YM4	0.32	0.38	37.6	19.5	21.0	2.5	9.7
	YM4 + YM3	1.08	1.63	89.1	22.2	10.0	ND[c]	9.9
	YM4 + YM3 broth	0.71	1.47	41.3	23.3	21.7	2.1	9.9
0.01	YM4	0.03	ND	2.0	0.2	ND	0.24	0.2
	YM4 + YM3	0.72	1.11	87.0	24.4	8.3	ND	9.9
	YM4 + YM3 broth	0.31	0.41	34.8	22.2	19.5	2.9	9.6
0	YM4	0.05	ND	ND	ND	ND	0.37	ND
	YM4 + YM3	0.47	0.58	57.6	26.3	13.3	1.4	9.8
	YM4 + YM3 broth	0.19	0.29	28.3	20.0	15.0	3.6	9.3

[a] Analyses were done 1 day after cellulose digestion was completed for grown cultures and after 30 days of incubation for nongrown cultures. All figures were corrected for the amounts contained in the inocula and the cell-free broth.
[b] OD, Optical density at 660 nm.
[c] ND, Not detectable.

DISCUSSION

Frequent contamination of cultures of *C. thermocellum* with glycolytic, thermophilic sporeformers such as *C. thermohydrosulfuricum* has been shown to be an obstacle for studies of *C. thermocellum* (5). To make matters worse, purification of *C. thermocellum* from contaminated cultures is not easy. Wiegel and Dykstra (21) succeeded in overcoming this difficulty by taking advantage of the strong adhesion of *C. thermocellum* to cellulose. In the present study, dark-field microscopy was used and found to work well in separating *C. thermocellum* YM4 from a stable coculture with *C. thermohydrosulfuricum* YM3.

Strain YM3 has the same morphological and physiological characteristics as other strains of *C. thermohydrosulfuricum* described by Hollaus and Sleytr (6) and Wiegel et al. (22), except that it ferments inulin. Its high ethanol yield is comparable to that of the most efficient performer of this species, strain E-39 (23). The 31.5 mol% guanosine-plus-cytosine content determined by the thermal denaturation method is slightly lower than the value (34.1 mol%) for strain JW102 obtained by Wiegel et al. (22). Properties of strain YM4 are very similar to the general description of *C. thermocellum* ATCC 27405 (2) and strains obtained by Ng et al. (15), but it is far more efficient in cellulose degradation than any other reported strains. The features different from those of other described strains are summarized in Table 3.

The faster cellulose degradation by the coculture than by the monoculture in the medium with 0.5% yeast extract is assumed to be caused by the quick removal of reducing sugars and a shift of the metabolism from high to low acid production by strain YM3, because reducing sugars are known to inhibit cellulase and because acids retard the growth of strain YM4 by lowering the pH of the medium. The data obtained by using the medium lacking yeast extract clearly show that the two bacteria depend on each other for nutrients needed for growth. Thus, it can be concluded that the relationship between strains YM3 and YM4 in cellulose fermentation under nutritionally unfavorable condition is one of mutualism, in which the former supplies a growth factor required by the latter and keeps the pH of the medium from dropping and, in turn, the latter provides the former with another growth factor and with fermentable sugars from cellulose.

Khan and Murray (7, 12) described intriguing relationships of a noncellulolytic saccharolytic anaerobe, *Clostridium saccharolyticum*, and two cellulolytic anaerobes, *Acetivibrio cellulolyticus* and *Bacteroides cellulosolvens*. Although those three bacteria were isolated from the same cellulose

TABLE 3. Unique properties of *C. thermocellum* YM4 in comparison with described strains

Property	Medium	Difference between strains:		Reference
		YM4	Described strain	
Spore formation	Cellulose	$<10^{-5}$	60–100% (JW20)	5
Carbohydrate utilization	Sucrose	+	Not reported	
	Sorbitol	+	+ (Icelandic), − (others)	19
CO_2/Na_2CO_3 requirement		Required	Not reported	
Doubling time (h)	Cellulose	2.7	6 (JW20), 7.2 (LQ8), 6.8 (N1), 6–17 (MC1–MC6)	5, 15, 15, 1 (respectively)
	Cellobiose	1.1	2.8 (JW20), 2.1 (N1)	5, 15
	Glucose	1.6	4 (JW20)	5
Avicelase (U/ml of broth)	Cellulose	0.22	0.026 (N1)	15
	Cellobiose	0.12	ND[a] (N1), 0.008 (ATCC 27405)	15, 17
CMCase (U/ml of broth)	Cellulose	2.21	0.66 (N1)	15
	Cellobiose	1.71	ND (N1), 0.20 (ATCC 27405)	15, 17

[a] ND, Not detectable.

enrichment culture and both of the cellulolytic bacteria produced the growth factor required by the saccharolytic bacterium, only *B. cellulosolvens* formed a stable coculture with *C. saccharolyticum*. It was indicated that mutual dependence was a decisive factor for the stability of the coculture: *B. cellulosolvens* relies on *C. saccharolyticum* for removal of toxic metabolite(s), but *A. cellulolyticus* derives no benefit from *C. saccharolyticum*. Similarly, coculture of strains YM3 and YM4 might be stabilized by the mutualism between them. However, another factor must exist for the establishment of their stable coculture, because they form a stable coculture even in medium containing a sufficient amount of yeast extract. The mutual support of strains YM3 and YM4 with growth factor(s) could be the force that enables them to grow in the natural environment, where sufficient nutrition is not always expected.

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