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Isolation of Cellulolytic Anaerobic Extreme Thermophiles from New Zealand Thermal Sites

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Avicel enrichment cultures from 47 thermal-pool sites in the New Zealand Rotorua-Taupo region were screened for growth and carboxymethyl cellulase activity at 75°C. Eight anaerobic cellulolytic cultures were obtained. The effect of temperature on carboxymethyl cellulase activity was measured, and bacteria were isolated from the five best cultures. Bacteria from two sources designated TP8 and TP10 grew at 75°C, accumulated reducing sugar in the growth medium and gave free cellulases with avicelase activity. Bacteria from sources designated Tok4, Tok8, and Wai21 grew at 75°C, accumulated no free sugars in the medium, and gave free carboxymethyl cellulases with virtually no avicelase activity. All were obligate anaerobic nonsporeforming rods which stained gram negative, grew on pentoses as well as hexoses, and gave ethanol and acetate as major fermentation end products. The isolated strain which produced the most active and stable cellulases (trivially designated TP8.T) had lower rates of free endocellulase accumulation at 75°C than did *Clostridium thermocellum* at 60°C, but its cellulase activity against avicel and filter paper in culture supernatants was comparable. Tested at 85°C, TP8.T carboxymethyl cellulases included components which were very stable, whereas *C. thermocellum* carboxymethyl cellulases were all rapidly inactivated. The TP8.T avicelase activity was relatively unaffected by Triton X-100, EDTA, and dithiothreitol. Evidence was obtained for the existence of unisolated, cellulolytic extreme thermophiles producing cellulases which were more stable and active than those from TP8.T.

Cellulose fermentations to industrial chemicals and fuels such as ethanol are possible long-term alternatives to dwindling fossil fuels and feedstocks. High-temperature fermentations utilizing thermophiles and extreme thermophiles are biologically and physically robust, with some potential technological advantages (34, 36). Interest has centered on anaerobic thermophilic cellulose fermentations by *Clostridium thermocellum* at temperatures up to 65°C (8, 34). *C. thermocellum* produces a cellulase complex capable of degrading crystalline celluloses and ferments the resulting sugars to ethanol and short-chain aliphatic acids. Defined coculture thermophilic cellulose fermentations of *C. thermocellum* with saccharolytic thermophiles (1, 23) and extreme thermophiles (34) can produce ethanol in high yield. Many studies on the nature of the *C. thermocellum* cellulase complex have been done (10, 14, 16, 26).

Studies aimed at utilization of *C. thermocellum* fermentation have involved (i) optimization of wild-type strains for growth, cellulase production, and action, giving up to fivefold increases in activity (12, 31), (ii) mutation and selection to improve cellulase yield (1, 23), and (iii) cloning of the cellulase genes into a suitable vector (14). Studies on *Trichoderma reesi* have followed a similar course (29). Selection of the most appropriate wild-type organism is critically important. There may be major advantages in obtaining cellulolytic extreme thermophiles, both to carry out cellulase fermentations at temperatures higher than 65°C and to yield genes specifying more stable, and perhaps more active, cellulase complexes than those of *C. thermocellum*. The existence of cellulolytic extreme thermophiles has been reported by Ljungdahl et al. (19), who obtained a culture of an Icelandic pool inoculum which degraded cellulose at 84°C.

The object of this study was to isolate extremely thermophilic anaerobic bacteria capable of degrading cellulosic materials at rates comparable to that of *C. thermocellum* but at 75°C. We screened 47 randomly selected thermal sites in the Rotorua-Taupo thermal area of New Zealand for such bacteria and isolated several strains.

A preliminary report of this work (32), as well as a further description of the physiology of one of the bacteria isolated (TP8.T) and its comparison with *C. thermocellum* (28), has appeared. The name *Caldocellum saccharolyticum* gen. nov., sp. nov. has been proposed for TP8.T (A. M. Donnison, C. M. Brockelsby, R. M. Daniel, and H. W. Morgan, submitted for publication), and the organism has been deposited with the American Type Culture Collection, Rockville, Md.

MATERIALS AND METHODS

Organisms and growth and maintenance procedures. *C. thermocellum* ATCC 27405 was used. The origin of the extreme thermophiles is described below.

Cultures were routinely grown in Universal or McCartney bottles with about 2 ml of head space (which increased after sampling) and were inoculated and sampled under an N_2 flow by modifications of the Hungate (11)-Bryant (5) technique. The medium used was basal mineral salts medium supplemented with trace elements and vitamins as described for *Thermoanaerobium brockii* (37) and contained 0.3% yeast extract and either 1% (or 0.5%) avicel 50 (Sigmacell 50; Sigma Chemical Co.) or 0.2% cellobiose. In initial enrichments, the medium also contained 1% Trypticase peptone (BBL Microbiology Systems) and 0.1% sodium thioglycolate

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was added as reductant. To reduce possible toxic effects, the reductant was changed to 0.1% filter-sterilized cysteine hydrochloride (added before autoclaving and followed by preconditioning at 65°C for 18 h) during isolation of TP8 strains and for all experiments on isolated bacterial strains. The medium was autoclaved for 30 min at 121°C. All strains were routinely maintained by subculture at 75°C and storage at 6°C. Difficulties in preservation by freeze-drying were overcome by including FeS (4).

Sampling of thermal sites. Samples of mud and biomass (if present) were taken from 47 pools in four thermal areas from the Taupo-Rotorua region of New Zealand, i.e., Tikitere, Waimangu, Taupo, and Tokaanu (abbreviated Tiki, Wai, TP, and Tok, respectively, in the site designations). These sites were selected for their accessibility and for temperatures above 70°C (with one exception) but were otherwise chosen randomly. They ranged from moderately dilute streams to the high-ionic-strength saline pools typical of Tokaanu with temperatures up to 97°C to the acid pools typical of Tikitere. They included hot spots in pools containing algal mats (which have a growth maximum of 65°C [3]), small pools containing considerable amounts of vegetation, and pools containing pieces of wood. Bacterial cultures were named after our accession number of their source, e.g., TP8.

The site temperature was recorded, and two bottles of clear pool water were also taken from suitable sites. Water pH and conductivity were immediately measured in one sample, and the second was reserved for subsequent mineral analysis that was carried out by the Water Section, Ministry of Works and Development, Hamilton. Samples for culture were taken in 28-ml Universal bottles which were completely filled and sealed tightly to maintain anaerobiosis. They were transported to the laboratory without temperature control and kept at 4°C until use. Wood samples were homogenized just before inoculation.

Isolation of cellulolytic extreme thermophiles. Bacteria were isolated from the third enrichment cultures of TP8, TP10, Tok4, Tok8, and Wai21 samples by using 10-fold dilutions in Universal bottles containing 27 ml of culture medium plus 1.5% molten Bacto-Agar (Difco Laboratories) and 0.5% MN 300 cellulose. A dilution series of TP8 was also made and was further supplemented with 0.5% cellobiose. The agar bottles were incubated at 70°C until colony formation occurred. At 75°C, the agar slowly melted. For further purification, 0.5% cellobiose replaced cellulose in the agar. To isolate individual colonies, a core was removed from the agar with a sterile 11-mm cork borer and sliced with a sterile scalpel into ca. 2-mm slices on a petri dish in a gas-filled (originally N_2, modified to CO_2) crystallizing dish under a stereoscopic microscope. An agar slice containing well-formed cellulose-clearing colonies was transferred to a fresh sterile petri dish under the gas flow, and a block of agar containing the colony was placed into a Universal flask containing warm prereduced medium and crushed to liberate the cells.

Only TP8 and TP10 yielded colonies able to produce clear zones in cellulose agar. Wai21, Tok4, and Tok8 yielded isolated bacteria with carboxymethyl cellulase (CMCase) activity but which did not accumulate reducing sugars in the medium (see Table 1). A primary enrichment culture from the homogenized wood from site TP8 yielded a culture which on agar dilution contained one very large clearing colony, well separated from two noncellulolytic colonies. The cellulolytic colony was subcultured in cellobiose medium and then reisolated from single colonies in cellobiose agar to reduce the possibility of coisolating different bacteria (1, 15). TP8 was reisolated from cellobiose agar three times and taken through seven successive 10-fold endpoint dilution series in liquid medium (cellobiose or Sigmacell 50), with transfer between series being performed during the logarithmic phase; the isolate was designated TP8.T.6.3.3.1. In subsequent studies, this isolate has been called simply TP8 (28) or *Caldocellum saccharolyticum* (Donnison et al., submitted). Two types of TP8 cellulolytic bacteria were subsequently isolated from different enrichment subcultures, including a 500-liter fermentation.

Because in-pool enrichment of cellulolytic bacteria seemed crucial for their successful isolation, an attempt was made to seed pool RT8 with pieces of cotton laboratory coat left for 12 weeks. RT8 was the outlet pool of a geothermal heat exchanger, with variable temperatures up to 105°C and with pH 8.9; it contained large amounts of wood and vegetation. A visible attack of the cellulose occurred, and the cotton cloth was covered with gray slime. Dilution into cellulose agar yielded clearing colonies. Twelve were cultured and showed differences in CMCase stability and rates of cellulose consumption. One, designated LC8, was much more thermally stable and active than TP8 and TP10 bacteria but rapidly lost cellulolytic activity during subculture, so that we were unable to reisolate it into stable culture.

Partial purification of TP8.T cellulase. The TP8.T cellulase was partially purified by ammonium sulphate precipitation (85% saturation) of 80 liters of culture supernatant after 21 days of growth, ultrafiltration, and washing of the redissolved enzyme by using a Millipore PTGC disk ultrafilter with a 10,000-molecular-weight cutoff.

Cellulase assays. Cellulases were measured in culture medium supernatants that had been removed from cells and cellulose by centrifugation at 15,000 × *g* for 10 min. Enzymes from *C. thermocellum* were incubated at 60°C, and those from the extreme thermophiles were incubated at 75°C. CMCase was estimated by using conditions and reagents as described for *C. thermocellum* (33). The production of reducing sugar was measured by the dinitrosalicylic acid method, modified to include 0.040 mM glucose in the color reagent to eliminate the initial lack of color response otherwise found in the glucose concentration curve (22). Under these conditions, one cellobiose molecule gave an absorbance equal to that of 1.7 glucose molecules.

Avicelase activity was measured in 1 ml of culture supernatant (or crude-enzyme concentrate) plus 1 ml of 0.1 M citrate buffer (pH 6.0) containing 2% avicel 50 (Sigmacell 50) after incubation at 75°C for the times shown. Reducing sugar was estimated by the dinitrosalicylic acid method after centrifugation (20,000 × *g* for 10 min) to remove residual avicel. Filter paper activity was measured similarly, with a 50-mg (1 by 6 cm) strip of Whatman no. 1 filter paper replacing the avicel (21). Activities are given in micromoles or nanomoles per minute per milliliter of culture supernatant.

General analytical techniques. Bacterial growth was estimated as visible turbidity above the unshaken cellulose layer. A simple scoring system was used, with an approximate doubling in turbidity between ranks. Protein was measured by a modification of the technique of Lowry et al. (27). Glucose was measured by using a commercial glucose oxidase (Boehringer Mannheim Biochemicals) and compared against appropriate glucose standards in anaerobic medium (which inhibited the assay). Fermentation end products were analyzed directly in culture supernatants with a Pye-Unicam model GCD chromatograph at 160°C, with a glass column (inner diameter, 1.8 by 4 mm) packed with

TABLE 1. Cellulase activities in cellulolytic primary enrichment cultures[a] grown at 75°C

Thermal site location	CMCase activity (nmol min^{-1} ml^{-1}) on day 6 of growth (60°C, 30 min)[b]	CMCase activity (nmol min^{-1} ml^{-1}) on day 21 of growth		Accumulated reducing sugar on day 21 of growth			Visible cellulose loss[c] on day 21
		60°C, 10 min[b]	75°C, 10 min[b]	mM	% of cellulose	% as glucose	
Wai21	104	218	273	5.7	9.5	42	+
Wai24	31	36	36	0	0		0
Wai25	65	115	125	0	0		0
Tok3	39	64	77	0	0		0
Tok4	58	111	134	0	0		0
Tok8	41	60	69	0	0		0
TP8	89	151	222	28.3	42.2	72	++
TP10	15	71	152	4.8	8.0	48	+

[a] Medium was basal salts–1% avicel 50–0.3% yeast extract–1% Trypticase peptone.
[b] CMCase incubation conditions.
[c] Symbols: 0, no loss; +, 1/3 loss; ++, 2/3 loss.

Chromosorb 101 (100/120 mesh), a carrier nitrogen flow of 40 ml min^{-1}, and flame-ionization detection.

RESULTS

Screening for cellulolytic extreme thermophiles. The 47 randomly chosen thermal pool water, sludge, and biomass samples were screened for cellulolytic bacteria by measuring cellulase activity in anaerobic, avicel enrichment cultures at 75°C. A total of 29 cultures grew, of which 8 subcultured to enrichments with CMCase activity (Table 1). In most cultures, CMCase was more active at 75°C than at 60°C. Sugar accumulation and visible cellulose attack occurred in three cultures, those from Wai21, TP8, and TP10. One source (Tok6) yielded a further aerobic cellulolytic culture which could not be maintained.

There were substantial differences in the mineral composition of water samples from the major sites (Table 2). The only obvious common features were the presence of decaying cellulosic biomass and relatively neutral pH conditions, similar to the initial pH of the enrichment medium. Inocula from pools which were acidic (e.g., the Tikitere sites) often grew but yielded no cellulolytic bacteria. To characterize cellulase thermal stability in these extreme-thermophile enrichments, we measured the temperature effect on CMCase activity (Fig. 1). Virtually all CMCases were maximally active in these 10-min assays at 80°C or above. Tok3, Wai24, and Wai25 isolates had relatively low or unstable activities and were not studied further.

Isolation of cellulolytic extreme thermophiles. Cellulolytic bacteria were successfully isolated from five sites. Isolates from Wai21, Tok4, and Tok8 produced thermally stable free CMCases but no avicelase activity. We plan to report separately on their properties. Only TP8 and TP10 yielded isolates which actively degraded avicel and accumulated reducing sugars in the medium. TP8.T was reisolated 10 times.

Two types of cellulolytic bacteria were finally isolated from TP8 enrichments, indicating that complex cellulolytic consortia were present after culture at 75°C (Table 3). Type 1, which included TP8.T, had moderate CMCase activity, showed avicelase activity, and accumulated considerable reducing sugar in the medium. The other type had over twice the level of CMCase, which was more stable at 85°C, showed no free avicelase activity, and accumulated low levels of reducing sugar in the medium. This second type of cellulolytic extreme thermophile from TP8 was intermediate in properties between the TP10/TP8-Type 1 strains and the Wai21, Tok4, and Tok8 isolates, which produced CMCase but accumulated no reducing sugar.

Growth requirements and the physiology of cellulase production. Isolates TP8.T and TP10 were rod-shaped (ca. 0.4 by 4 μm) obligate anaerobes staining gram negative. No terminal spores were ever found. Both grew on glucose, celloboise, xylose, and xylan as well as cellulose as the carbon source (in 0.3% yeast extract). The major fermentation products on cellulose were ethanol and acetate. After 2 weeks of growth, cultures supplemented with 0.2% cellobiose had half the CMCase activity of cellulose-only

TABLE 2. Characteristics of pools from which cellulolytic bacteria were isolated

Site	Temp (°C)	pH at 25°C	Conductivity (mho)	Organic matter[a]	Na^+ (mM)	K^+ (mM)	Ca^{2+} (mM)	Mg^{2+} (mM)	NH_4^+ (mM)	Cl^- (mM)	SO_4^{2-} (mM)	HCO_3^- (mM)	SiO_3^{2-} (mM)	PO_4^{3-} (μM)	NO_3^- (μM)	Na^+/K^+	Na^+/Ca^{2+}	NH_4^+/NO_3^-
TP10	78	6.8	570	++	1.04	0.07	0.05	0.017	1.12	1.16	0.07	0.61	0.09	0.68	4.46	16	22	241
TP8[b]	48			+++														
Tok8	75–80 (gradient)	5.6	8,480	+	78.4	3.3	0.85	0.012	0.16	72.0	0.08	0.51	1.7	4.0	86.3	24	92	2
Tok4	89	5.6	7,050	+++	60.5	7.3	1.2	0.04	0.86	54.0	1.3	0.25	1.6	2.6	11.6	8	52	74
Wai21	82	6.3	5,020	+	13.7	1.0	0.45	0.19	0.25	10.8	2.1	0.13	1.5	0.36	3.35	14	30	75
Wai24	70	7.7	5,730	?	21.2	1.9	0.90	0.18	0.06	21.1	1.9	0.004	1.85	2.87	10.2	11	24	6
Wai25	67	6.8	3,950	?	16.2	0.79	0.77	0.30	0.001	13.9	1.3	0.002	1.92	3.33	18.2	21	21	0.05
RT8	75–105 (variable)	8.9	Variable	+++	25.7	0.95	0.04	0.003	0.01	13.8	0.7	8.7	2.6	1.9		27	695	

[a] Organic matter was rated on a scale up to +++ for solid biomass.
[b] TP8 was the decomposed end of a *Pinus radiata* plank downstream from site TP10.

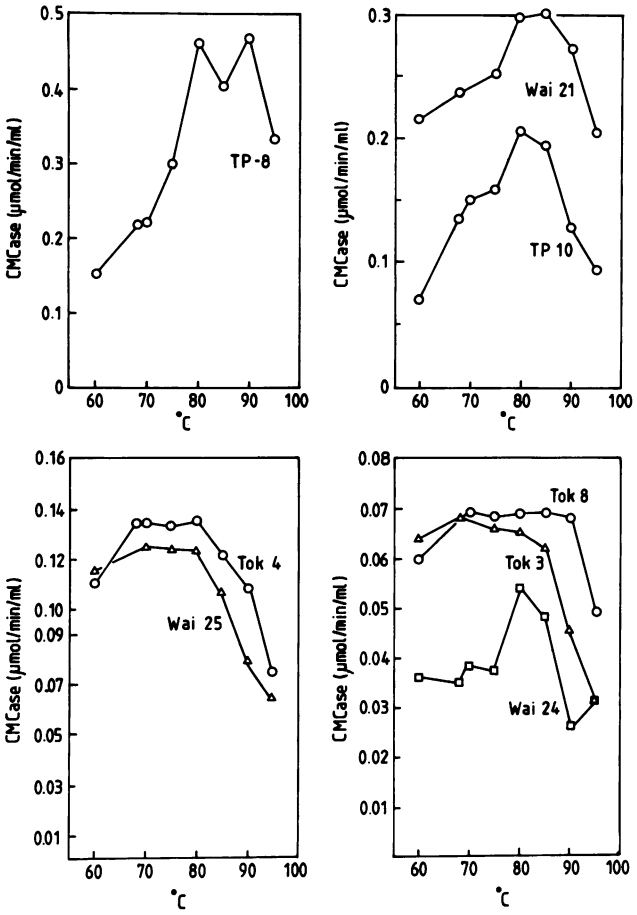

FIG. 1. Temperature dependence of CMCase reaction rates in cellulolytic enrichment cultures. Supernatants were removed from subcultures of the primary enrichments at day 20 of growth and assayed for CMCase in 10-min incubations at the temperatures shown.

TABLE 3. CMCase stability and reducing sugar accumulation in isolated TP8 bacteria[a]

Isolation series	No.	CMCase[b] (nmol·min^{-1}·ml^{-1})	CMCase stability[b]		Reducing sugar accumulation[c] (mM)
			After 1 h at 85°C (% remaining)	After 3 h at 85°C (% remaining)	
TP8.T, TP8.B	11	47 ± 6	24 ± 2	18 ± 1	7.3 ± 0.4
TP8.A, TP8.C	4	160 ± 7	53 ± 2	39 ± 1	1.5 ± 0.4

[a] Values are given as mean ± standard error of the mean.
[b] At day 10 of cultures grown at 75°C.
[c] At day 21 of culture.

cultures. *C. thermocellum* was unaffected by 0.2% cellobiose supplementation.

CMCase and reducing-sugar levels during growth in 1% avicel medium for TP8.T and TP10 at 75°C and for *C. thermocellum* at 60°C are shown in Fig. 2. The pH fell from 7 to between 5.5 and 6 after 9 days of growth, possibly limiting growth. The maximum visible turbidity in *C. thermocellum* (reached on day 5) was about four times the maximum turbidity seen in the extreme thermophiles but subsequently decreased. Free CMCase accumulated in all cultures between days 2 and 14 (late-exponential and early-stationary phase) and decreased by about 50% over the following 14 days. *C. thermocellum* cultures accumulated reducing sugar before day 5, but TP8.T and TP10 cultures did so only after day 5, having accumulated 15 to 25% of the *C. thermocellum* level by day 27.

Mesophilic cellulolytic bacteria are generally unaffected by wide variations in medium mineral composition (11). Accordingly, we chose as standard a medium used for isolating extreme thermophiles (37). *C. thermocellum* is generally grown in a derivative of medium CM-3 which has, in particular, much higher levels of Mg^{2+}, Ca^{2+}, and sulfate (24). We examined the growth of our strains in a derivative of this medium, GS-2 (13), commonly used for *C. thermocellum* studies. It precipitated at 75°C, TP8.T and

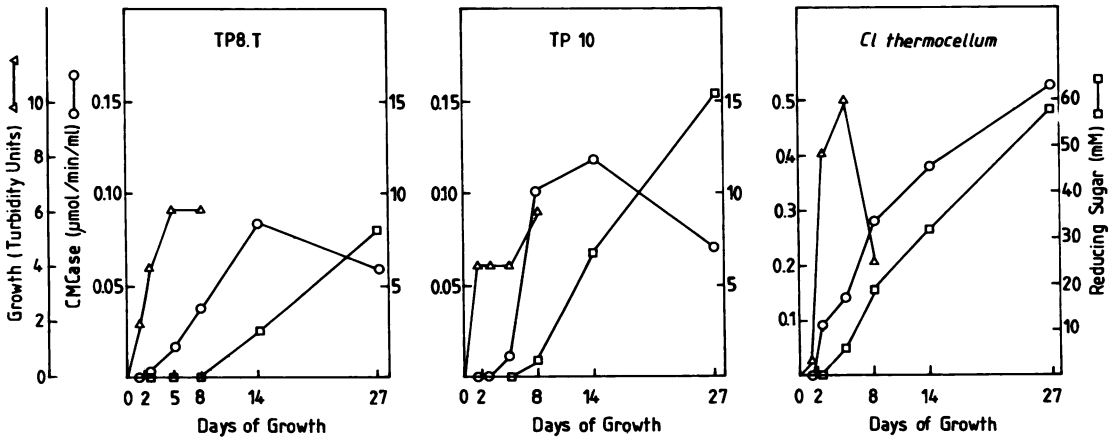

FIG. 2. Time course of endocellulase and reducing-sugar accumulation. Cultures (500 ml) of TP8.T and TP10 were grown at 75°C and *C. thermocellum* was grown at 60°C in 1% Avicel 50–0.3% yeast extract–basal salts medium including trace elements and vitamins. At the times shown, turbidity was assessed (see Materials and Methods), portions were removed and replaced by N_2, and CMCase and reducing-sugar levels were measured.

TP10 grew only moderately, and Wai21 and Tok8 strains with incomplete cellulases grew poorly or not at all. At 60°C, *C. thermocellum* grew well. It appears that for cellulolytic extreme thermophiles, the mineral composition of the medium may have a pronounced effect on growth.

Cellulase thermal stability. TP8.T and TP10 CMCases were fairly stable during culture at 75°C (Fig. 2). Together with *C. thermocellum* CMCases, they were examined for stability at 85°C at pH 7.0 (Fig. 3). *C. thermocellum* CMCases lost all activity in 5 min. Both TP8.T and TP10 had unstable cellulase components making up about 50% of the total. The activity then decreased with a half-life of about 1 h. TP8.T cellulase also stabilized or was activated during the 30 min following the initial decrease and had 20% residual activity after 18 h at 85°C (not shown). TP10 cellulase was completely inactivated after 4 h. These bacteria therefore had cellulase components of varying thermal stability, mostly very stable compared with cellulases from *C. thermocellum*. The cellulase from the RT8 enrichment culture (LC8) was active in degrading avicel and was exceptionally stable at 85°C compared with TP8.T.

Detergent, thiol and EDTA effects on cellulase. Addition of the neutral detergent Triton X-100 (2%) to a TP10 mixed-enrichment culture had no effect on the CMCase reaction (Fig. 4). Triton X-100 caused only a very slight loss in TP8.T avicelase activity in a partially purified enzyme preparation, but sodium dodecyl sulfate (1%) caused a 90% loss. Dithiothreitol and EDTA had no effect. There was no reaction at pH 10 (data not shown).

Comparison of cellulase activities with those from *C. thermocellum*. Avicelase filter paper activity, CMCase activity, and protein concentration from culture supernatants of stationary-phase TP8.T, TP10, and *C. thermocellum* are shown in Table 4. *C. thermocellum* produced higher free CMCase activities per milliliter of culture supernatant than did TP8.T and TP10. The avicelase (and filter paper activity)-to-CMCase ratios from extreme thermophiles were higher than those from *C. thermocellum*.

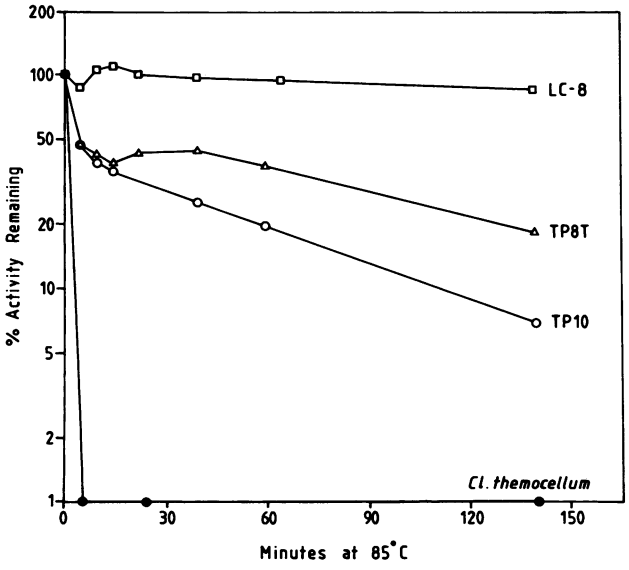

FIG. 3. CMCase stability at 85°C of TP8.T, TP10, LC-8, and *C. thermocellum*. Culture supernatants were removed from stationary-phase cultures grown for 4 weeks at 70°C (TP8.T, TP10, and LC-8 enrichment culture) or 60°C (*C. thermocellum*). Growth was in 1% Avicel 50–0.3% yeast extract–basal salts medium (except for TP10, which was grown on 0.5% cellobiose instead of 1% Avicel 50). The supernatants were preincubated at 85°C in 0.05 M citrate-phosphate buffer (pH 7.0), and residual CMCase activity was measured at 75°C (60°C for *C. thermocellum*). The initial activities (in nanomoles per minute per milliliter of supernatant) were as follows: TP8.T, 89; TP10, 73; LC8, 88; *C. thermocellum*, 187.

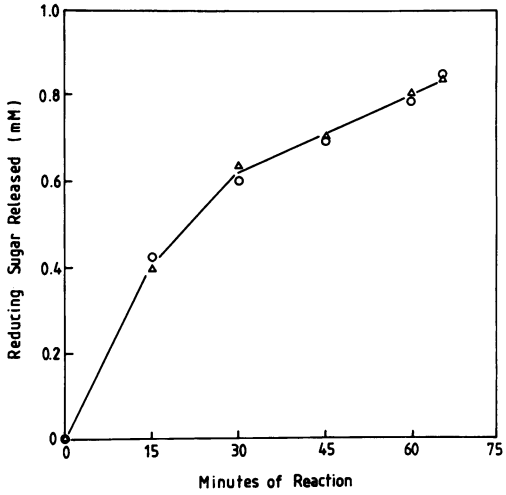

FIG. 4. Effect of Triton X-100 on CMCase yield and reaction rate in a TP10 mixed culture. A TP10 enrichment culture was divided, Triton X-100 (final concentration, 2%) was added to one portion, and supernatants were prepared. CMCase activity at 60°C was measured with (△) and without (○) Triton X-100.

DISCUSSION

The main characteristic common to thermal sites yielding cellulolytic bacteria was the presence of decaying organic matter, at least in the samples taken. These sites are natural enrichment systems for cellulolytic bacteria and, even at extreme temperatures, potentially give a number of bacterial species in complex ecosystems. Isolation of two types of cellulolytic bacteria from the TP8 sample, which was a decomposing *Pinus radiata* plank, supports this supposition. Natural and artificial in-pool enrichment on degrading biomass is a powerful technique for concentrating cellulolytic bacteria and may be a prerequisite for their successful isolation. It is clear that very active cellulolytic

TABLE 4. TP8.T, TP10, and *C. thermocellum* cellulase activities[a]

Strain	Cellulase activity[b] (mU · ml^{-1})[c]:			Ratios		Total supernatant protein (μg · ml^{-1})
	CMCase[d]	Avase[e]	FPase[e]	Avase/CMCase	FPase/CMCase	
TP8.T	50	4.3	2.7	0.09	0.05	30.1
TP10	18	4.6	2.5	0.25	0.14	28.3
C. thermocellum	218	9.2	3.2	0.04	0.01	69.1
None (medium only)						17.7

[a] Measured in supernatants from cultures grown for 7 days at 75°C in 1% avicel 50–0.3% yeast extract–basal salts medium.
[b] Incubation temperature was 75°C for TP8.T and TP10 and 60°C for *C. thermocellum*.
[c] U, Micromoles per minute.
[d] A 30-min incubation was used.
[e] A 2-h incubation was used. Abbreviations: Avase, avicelase; FPase, filter paper activity.

extreme thermophiles which could not be isolated in stable culture were present in pool RT8 and presumably in other pools, as the sites sampled made up only a small fraction of those available.

Further studies are needed to clarify the taxonomic position of the cellulolytic extreme thermophiles. Although the cells stained gram negative, electron-microscopic studies have shown that the cell wall of TP8.T has a gram-positive structure (28). As terminal spores were never seen, it is unlikely that these isolates are clostridia. Metabolically, they are closer to *Clostridium stercorarium* (20) than to *C. thermocellum* in fermenting a wide range of sugars and in growth on xylose and xylan. DNA-DNA hybridization has confirmed the lack of relationship between these isolates and *C. thermocellum*, and G+C ratios distinguish them from *C. stercorarium*. These data suggest that these organisms belong to a new genus (Donnison et al., submitted).

Extreme-thermophile cellulases were thermally stable. The most stable bacterial cellulases yet reported appear to be those of a *Thermoactinomyces* species (9), *C. thermocellum* with a temperature optimum of 70°C (13, 18, 24), and *C. stercorarium* (20). Except for *C. stercorarium* endocellulase, which has 50% residual activity after 1 h at 85°C but does not have a free avicelase, the extreme thermophile cellulases are considerably more stable. The rate of enzyme inactivation at 85°C, which we used as a criterion of thermal stability, is fairly stringent, as shown by the very rapid inactivation of *C. thermocellum* CMCases (Fig. 3).

Extreme-thermophile cellulases consist of a mixture of relatively heat-stable and unstable CMCase components. TP8.T cellulase showed a period of stable activity before partial inactivation to a very stable 20% residual activity. This activation, inactivation, and temporary stabilization phenomenon appeared to be sensitive to ionic conditions such as the buffers used (C. H. Sissons and K. R. Sharrock, unpublished data). It is probably a consequence of the known complexity of bacterial cellulase systems. Studies of the TP8.T cellulase system (32; K. R. Sharrock, D. Phil. thesis, University of Waikato, Hamilton, New Zealand, 1985) suggest that it has a number of multiple cellulase activities possibly comparable to those from *C. thermocellum* (10, 14, 16), *Ruminococcus albus* (35), and *Cellulomonas fimi* (17). This complexity probably also accounts for some of the flat curves seen in studying the effects of temperature on CMCase activity, e.g., in Wai24 and Tok8 isolates in Fig. 1.

Enzymes from extreme thermophiles also tend to be more stable than those from mesophiles to detergent denaturation (6) and protease attack (7). Triton X-100 had virtually no effect on CMCase activity in a TP10 enrichment culture (possibly containing several different cellulolytic bacteria) at 75°C or on TP8.T avicelase activity. SDS inactivation of TP8.T cellulase was incomplete. Thus these enzymes are relatively detergent resistant.

A cellulase with a high activity against crystalline cellulose is desirable, e.g., for selecting potential cellulase genes for enhancement by genetic engineering. While the CMCase assay is rapid, convenient, and accurate (and a valid screening test), the usefulness of a cellulase rests on its activity against crystalline and native celluloses. Literature comparisons of cellulase activities are dubious because heterogeneous nonstandard substrates and differing assay conditions are used (21). All short-term assays of cellulase on natural celluloses must be interpreted very cautiously when being extrapolated to rates of long-term effective cellulosic degradation because more easily degraded amorphous areas are degraded before the heterogeneous, less accessible crystalline regions (13). The ratio of free to cellulose-bound cellulase in culture will also vary with cellulose type and concentration and phase of growth (2). We tried in part to overcome these difficulties in our initial studies by using the exact assay for CMCase described by Weimer and Zeikus (33) (except for assaying at 75°C rather than 60°C) and by growing *C. thermocellum* and comparing its activity at 60°C, as a bench mark, directly with our assays of extreme thermophiles at 75°C.

The CMCase level which we obtained for *C. thermocellum* in medium CM-3 (200 to 500 nmol · min^{-1} · ml^{-1}) agrees well with the 300-nmol · min^{-1} · ml^{-1} maximum value obtained by Weimer and Zeikus (33). Our avicelase and filter paper activities compare well with those obtained by Ng and Zeikus using similar assays (25). TP8.T grown in our standard extreme-thermophile medium gave lower CMCase levels of 50 to 100 nmol · min^{-1} · ml^{-1}, and TP10 gave levels of 20 to 120 nmol · min^{-1} · ml^{-1}. However, the free activity against crystalline cellulose of TP8.T and TP10 appeared to be comparable to that of *C. thermocellum*, with less extracellular protein produced. The ratio of avicelase to CMCase was severalfold higher in the TP8.T and TP10 strains than in *C. thermocellum*. For TP8.T, when total (free plus bound) cellulase levels are measured (28), the avicelase-to-CMCase ratio is about fivefold greater than for *C. thermocellum*. These data suggest that extreme thermophiles have cellulase activities, acting on crystalline celluloses, at least comparable to those from *C. thermocellum*.

The specific activities of complete cellulases from fungi and bacteria seem to be similar, with most species differences being related to special properties (e.g., feedback inhibition or presence of β-glucosidases) and to the amount of enzyme protein produced (30). Since cellulase instability is regarded as an important limitation in potential industrial enzymatic cellulase hydrolysis strategies (29), cellulolytic extreme thermophiles may provide exceptionally advantageous sources of cellulases and organisms for use in such programs.

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