Carboxymethylcellulase Produced by Facultative Bacteria from the Hind-gut of the Termite *Reticulitermes hesperus*

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Bacillus cereus RW1 and *Serratia marcescens* RW3, isolated from the hind-gut of the termite *Reticulitermes hesperus*, both grew well on mesquite wood and produced moderate amounts of carboxymethylcellulase. Carboxymethylcellulose (CMC) gels were depolymerized rapidly by *B. cereus* RW1 and slowly by *S. marcescens* RW3. The depolymerization of CMC was pH and temperature sensitive. Depolymerization of gels by growing cultures of *B. cereus* RW1 and the action of cell-free extracts of *B. cereus* RW1 on CMC sols were optimum at pH 6.0 and 5.5, respectively. Glucose and cellobiose increased the rate of CMC gel depolymerization. Enzyme synthesis rather than growth was stimulated by the addition of glucose to a culture of RW1 growing on a non-cellulosic substrate. *Bacillus cereus* RW1 produced both cell-free and cell-bound carboxymethylcellulase.

INTRODUCTION

The isolation of bacteria from the hind-gut of the termite *Reticulitermes hesperus*, their growth on mesquite wood and the depolymerization of carboxymethylcellulose were reported by Thayer (1976). Since wood contains many potential substrates, growth did not necessarily indicate cellulolytic activity. During growth *Bacillus cereus* RW1 and *Serratia marcescens* RW3 solubilized 2 to 5% more mesquite wood than could be achieved by autoclaving, which solubilized 23%. Although *B. cereus* RW1 and *S. marcescens* RW3 attacked the insoluble components of the wood, they preferred the soluble substrates. Some cellulose hydrolysis and distinct carboxymethylcellulose depolymerization indicated weak cellulolytic activity. Since these organisms were isolated from the hind-gut of the termite and grew on wood, they might contribute to cellulose digestion in the termite. This study characterized the carboxymethylcellulase activity of these organisms.

METHODS

Organisms. *Bacillus cereus* RW1 and *Serratia marcescens* RW3, obtained as described previously from the hind-gut of *Reticulitermes hesperus* (Thayer, 1976), were maintained on BBL Trypticase Soy Agar (TSA).

Media. The basal medium used to study growth on wood contained (g l⁻¹ in distilled water): NaCl, 3.0; KH₂PO₄, 1.0; K₂HPO₄, 1.0; (NH₄)₂SO₄, 2.0; MgSO₄, 0.05; CaCl₂, 0.05; Difco yeast extract, 0.50; and dormant mesquite wood sawdust, 10.0; the pH was adjusted to 6.5 after autoclaving. To determine the temperatures supporting maximum growth rates, cultures were grown in mineral salts medium containing 5 mM-glucose at pH 6.5 (the medium was sterilized by filtration).

Incubation and inoculation. Cultures were routinely grown at 35 °C in 100 ml medium in 500 ml baffled Erlenmeyer flasks shaken at 250 rev. min⁻¹ on a gyratory shaker. In some experiments, flasks with an attached 10 mm diam. cuvette were used; the design of these limited the volume of medium to 80 ml. Inocula were 0.1 ml per 100 ml medium.

Measurement of growth. Absorbance measurements were made at 420 nm in colourless media or at the

less sensitive 660 nm in yellow media. Viable cell numbers (colony-forming units) were determined by standard pour-plate count procedures with five replica TSA pour plates per dilution. After maximal development at the appropriate dilution, the colonies on all five Petri plates were counted. Cell protein was measured by the Lowry procedure (Herbert, Phipps & Strange, 1971).

Carboxymethylcellulase. Carboxymethylcellulase activity was estimated (i) by the release of reducing sugar, (ii) viscometrically, and (iii) by the rate of hydrolysis of carboxymethylcellulose (CMC) gels. The CMC reagent of Miller *et al.* (1960) was modified to contain (g l⁻¹): KH₂PO₄, 13.61; K₂HPO₄, 17.42; sodium carboxymethylcellulose (Sigma no. 8758), 10.0; glucose, 0.10; and thimerosal (ethyl mercurithiosalicylate; Sigma), 0.10; the pH was adjusted to 6.9. A 4 ml enzyme sample was mixed with 2 ml CMC reagent and incubated at 35 °C in a sealed 50 ml Erlenmeyer flask in a gyratory water-bath shaker. After incubation, duplicate 2 ml samples were withdrawn, mixed with 3 ml dinitrosalicylic acid reagent (Miller *et al.*, 1960), and held at 100 °C for 15 min before determining A₆₄₀. A standard curve was established with glucose.

Carboxymethylcellulose depolymerization was measured viscometrically at 34.9 ± 0.05 °C using an Ostwald viscometer with a water efflux time of 22.3 s at this temperature. One unit of activity was defined as the decrease in the time taken (in seconds) for the efflux of the incubation mixture per minute of incubation (Hulme & Stranks, 1971). Six efflux times were determined at 5 min intervals; the enzyme dilution was chosen so that the differences between successive readings were 5 s or less (Hulme, 1971). The standard procedure consisted of mixing 0.1 ml cell-free culture with 2.9 ml 0.2 M-potassium phosphate or 0.2 M-sodium acetate buffer and 5 ml CMC solution [containing 0.5 g CMC type 7HF (see below) and 0.01 g thimerosal per 100 ml, and stabilized according to Hulme (1971)]; 5 ml of this mixture was added to the viscometer. The thimerosal did not inhibit enzyme activity. The decreases in efflux time at 34.9 °C per min of incubation between successive measurements from 5 to 20 min were averaged.

CMC depolymerization was also detected by the liquefaction of gels containing sodium carboxymethylcellulose (Hercules Inc., Wilmington, Delaware, U.S.A.), either type 7HF which has a degree of substitution (DS) of 0.7 and a degree of polymerization (DP) of 3200, or type 7LF which has a DS of 0.7 and a DP of 400. The medium contained (g l⁻¹): CMC, 20, 25 or 60, as indicated in text; phytone (BBL), 10.0; NaCl, 5.0; and tap water. The pH was adjusted to 7.0 and the medium was dispensed in 16 × 150 mm test tubes and sterilized. In some experiments, the gel was buffered with 0.1 M-potassium phosphate to various pH values, measured after sterilization. Tubes were inoculated with 0.05 ml culture. The depolymerization proceeded from the surface towards the bottom of the gel with zero-order kinetics, reducing the gels to a water-like viscosity. Gel hydrolysis was measured at 2 d intervals for 16 to 20 d. Hydrolysis rates of CMC media were always compared in the same experiment at different pH values or temperatures. Rates of hydrolysis were determined by a least squares analysis of all measurements in which less than 100% of the gel was hydrolysed (Snedecor, 1956).

Location of carboxymethylcellulase activity. Strains were cultivated for 24 h in BBL Trypticase Soy Broth (TSB) without glucose. The bacteria were harvested by centrifuging at 10000g for 30 min and the supernatant fluid was retained for extracellular enzyme analysis. The bacteria were suspended in 0.1 M-potassium phosphate buffer pH 7.0 and a sample of the suspension was disrupted ultrasonically at 20 kHz using a Bronwill Scientific Biosonik Probe.

Catabolite repression of CMC depolymerase. Four identical cultures of *B. cereus* RW1 were grown in TSB without glucose; after 8 h, glucose was added to two of the cultures. At various times, A₆₆₀ was monitored; 10 ml samples were removed from each flask and chilled immediately to 4 °C for protein and enzyme analyses; and 2 ml samples were removed for analysis of whole culture CMC depolymerase activity.

Avicelase. The substrate for the analysis of Avicelase activity (C₁-cellulase) consisted of 5 g Avicel pH 101 (FMC Corporation, Newark, Delaware, U.S.A.) in 100 ml 0.1 M-potassium phosphate buffer pH 7.0. One ml substrate was incubated with 1 ml enzyme for 2 h at 35 °C, and the amount of reducing sugar released was determined as described above. All values were corrected for endogenous reducing sugar.

All experiments were duplicated.

RESULTS

Carboxymethylcellulase was produced maximally in the late-exponential or stationary phases of growth by strains of *B. cereus* RW1 and *S. marcescens* RW3 growing on a mesquite wood carbon and energy source (Table 1). Little change occurred in the medium reducing sugar concentration. No cell-free enzymic hydrolysis of Avicel was detected.

An Arrhenius plot of growth rates (Fig. 1a) indicated that the optimum temperature for growth of *B. cereus* RW1 in a glucose/mineral salts medium was between 30 and 40 °C with an observed maximum at 35 °C. The temperatures at which strain RW1 caused maximum rates of depolymerization of various CMC type 7HF gels were: 2% gel, 40 °C; 2.5% gel,

Table 1. Growth and carboxymethylcellulase activities of cultures of strains RW1 and RW3 growing on mesquite wood

Strain	Incubation time (h)	$10^{-7} \times$ Colony-forming units ml^{-1}	Reducing sugar ($\mu\text{g ml}^{-1}$)	Cell-free carboxymethylcellulase*
RW1	0	0.0036	554	18.7
	6	1.23	562	3.8
	10	ND	500	232
	25	12.6	400	154
	53	11.4	385	60.0
RW3	0	0.161	569	57.7
	6	29.8	510	0
	10	285	431	20.3
	25	369	523	89.9
	53	296	431	1.5

ND, Not determined.

* Activities are expressed as μg reducing sugar produced in 24 h by 1 ml cell-free culture broth.

Fig. 1. Arrhenius plots of the rates of liquefaction of CMC type 7HF gels by *B. cereus* RW1: (a) ●, 2.0% gel; (b) ○, 2.5% gel; ●, 2.5% gel containing 0.5% glucose; □, 2.5% gel containing 0.5% cellobiose; (c) ○, 6.0% gel; ●, 6.0% gel containing 0.5% glucose.

Also shown in (a) is an Arrhenius plot of *B. cereus* RW1 growth rates (○). Duplicate cultures were incubated in a water bath and agitated at 300 rev. min⁻¹. A_{420} for each culture was recorded at 30 min intervals for 24 h. Measurements were made at 5 °C intervals from 20 to 50 °C, but no growth (NG) occurred at 50 °C.

Fig. 2. CMC depolymerase synthesis during batch culture of *B. cereus* rw1 in Trypticase Soy Broth: ○, without glucose (cultures 1 and 2); ●, 5 ml of a 20% (w/v) filter-sterilized glucose solution added before sampling at 8 h, as indicated by the arrow (cultures 3 and 4). [If the enzyme were repressible, CMC depolymerase activity would continue to increase in cultures 1 and 2 after 8 h but not in cultures 3 and 4.] CMC depolymerase activities are expressed as units (see Methods) per ml culture. Cell protein: □, TSB without glucose; ■, TSB with glucose. Culture absorbance at 660 nm: △, TSB without glucose; ▲, TSB with glucose; no further readings were taken once the A_{660} of both cultures exceeded 1.7.

30 °C; 6.0% gel, 45 °C; 2.5% gel containing 0.5% glucose, 35 °C; 2.5% gel containing 0.5% cellobiose, 40 °C; 6.0% gel containing 0.5% glucose, 40 °C (Fig. 1). The depolymerization rate increased when either glucose or cellobiose was added to the medium (Figs 1b, c).

Similar results were obtained with *S. marcescens* rw3, except that it was less active than *B. cereus* rw1. The optimum temperature for depolymerization of a 2.0% CMC type 7HF gel was 30 °C with 8 mm of the gel degraded in 20 d. When 0.5% glucose was added to the same gel, the optimum shifted to 35 °C and 47.5 mm was depolymerized in 15 d. The same 35 °C optimum was identified for the depolymerization of 6.0% CMC type 7HF gels; 5.5 mm was depolymerized in 20 d in the absence of glucose and 13 mm in its presence. The optimum temperature for the growth of rw3 in the glucose/mineral salts medium was 40 °C.

Rates of CMC depolymerization by rw1 or rw3 were maximum at pH 6.0: in 10 d at this pH, rw1 depolymerized 47 mm of a 2.5% CMC (type 7HF) gel and rw3 depolymerized 17 mm. The rate of CMC gel liquefaction by *B. cereus* rw1 increased linearly from 0.2 to 4.9 mm per day as the pH of the medium was changed from 8.0 to 6.0; the rate decreased at pH 5.0. The rate of CMC gel liquefaction by *S. marcescens* rw3 was 0.2 mm per day at pH 6.5 but 1.2 mm per day at pH 6.0. A pH of 5.5 was optimum for depolymerization of CMC type 7HF by crude rw1 cell-free enzyme preparations. There was an 86% linear increase in activity between pH 7.5 and 5.5 and a 50% increase between pH 4.0 and 5.5 in phosphate buffers. Uninoculated controls were unaltered at the end of the experiment.

When glucose was added to a growing culture in TSB, CMC depolymerase activity increased approximately 1.8-fold. No such increase in absorbance or cell protein was observed (Fig. 2). Addition of glucose to crude cell-free preparations did not increase CMC depolymerase activity.

Carboxymethylcellulase activities in cell-free, whole-cell and broken-cell preparations are

Table 2. Carboxymethylcellulase activities in cell-free culture broth, whole-cell suspensions and broken-cell suspensions of strains RW1 and RW3

Strain	Cell protein ($\mu\text{g ml}^{-1}$)	Carboxymethylcellulase*			Ratio Cell-free Whole-cell
		Cell-free culture broth	Whole-cell suspension	Broken-cell suspension	
RW1	600	71.0	11.1	51.0	6.4
RW3	400	0	0	66.5	—

* Activities are expressed as μmol glucose produced in 24 h per mg cell protein.

shown in Table 2. *Bacillus cereus* RW1 had both cell-free and cell-bound activities. *Serratia marcescens* RW3 had only cell-bound activity that was expressed only after the cells had been disrupted ultrasonically. Neither culture produced demonstrable Avicelase activity.

DISCUSSION

Bacillus cereus RW1 and *S. marcescens* RW3 grew well on mesquite wood and produced carboxymethylcellulase activity which was related to the age and cell density of the cultures. It is not known what role carboxymethylcellulase may have in the actual hydrolysis of wood especially in the hind-gut of a termite.

The use of CMC gels for detection of pH and temperature optima for depolymerization proved valuable. The optimum temperature for growth corresponded well with that for the hydrolysis of CMC gels. The gel technique gave similar results to enzymic analysis and thus was valuable for surveying the CMC depolymerization activities of bacterial cultures. Its relative simplicity makes the gel technique ideal for comparing several cultures.

Cellobiose is a competitive inhibitor of the cellulase activity of *Trichoderma* (Halliwell, Griffin & Vincent, 1972). The unexpected increase in CMC depolymerization rates in the presence of glucose or cellobiose made it imperative to assay the effects of adding carbohydrates under conditions where growth effects could be separated from enzymic activity. The addition of glucose to cultures of *B. cereus* RW1 in TSB increased production of CMC depolymerase without increasing bacterial protein. The synthesis and activity of the CMC depolymerase in *B. cereus* RW1 does not appear to be subject to the usual end-product regulation. Instead, its synthesis was enhanced by the sugar, since adding glucose before sampling at 8 h caused neither immediate nor delayed increases in enzymic activity. This was confirmed in a separate study.

In *Myrothecium verrucaria*, Hulme & Stranks (1971) observed that cellulase was produced when glucose was used as a carbon source in rate-limiting amounts, and concluded that cellulase in *M. verrucaria* was constitutive and that its production was partially controlled by catabolite repression. Since carboxymethylcellulase and CMC depolymerase were produced by RW1 and RW3 in the absence of any cellulosic materials, the enzyme(s) must be constitutive. The increased synthesis of CMC depolymerase by RW1 in the presence of glucose indicated possible enzyme induction by this substrate. Suzuki, Yamane & Nisizawa (1969) noted the formation of both cell-bound and extracellular cellulase by *Pseudomonas fluorescens* var. *cellulosa* when glucose, cellobiose, sophorose or cellulose was the carbon source. Sophorose and cellulose stimulated the synthesis of extracellular cellulase. Sophorose also induced cellulase synthesis in *Trichoderma viride* (Mandels, Parrish & Reese, 1962) and *P. fluorescens* var. *cellulosa* (Yamane *et al.*, 1970). Mandels *et al.* (1962) demonstrated that sophorose was a contaminant of some commercial preparations of glucose. The reagents used in this study were of the highest commercial purity but may have contained some sophorose. Mandels & Reese (1960) reported that high concentrations (0.5 to 1.0 %) of cellobiose or other rapidly

metabolized carbon sources strongly repressed cellulase formation by *T. viride*, and addition of glucose or cellobiose to a culture producing cellulase (carboxymethylcellulase) inactivated the enzyme already formed. The conditions used in this study were predicted to inactivate or to stop synthesis of cellulase. The regulation of cellulase synthesis in both *B. cereus* RW1 and *S. marcescens* RW3 appears to differ from the fungal and bacterial systems described above and should be investigated further.

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