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Phenotypic Characterization of Polysaccharidases Produced by Four *Prevotella* Type Strains

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Abstract. Four ruminal *Prevotella* type strains, *P. ruminicola* JCM8958^T, *P. bryantii* B_14^T, *P. albensis* M384^T, and *P. brevis* ATCC19188^T, were characterized for polysaccharide-degrading activities with the reducing sugar release assay and zymogram analyses. Carboxymethylcellulase, xylanase, and polygalacturonate (PG)-degrading enzyme activities were determined in cultures grown on oat spelt xylan, xylose, arabinose, cellobiose, and glucose as sole growth substrates. *P. ruminicola* and *P. albensis* showed carboxymethylcellulase induction patterns. When xylan was supplied as a sole growth substrate, xylanase activities produced by *P. bryantii* and *P. albensis* were at least 18- and 11-fold higher, respectively, than during growth on other carbohydrates, suggesting that the regulation of the xylanases was highly specific to xylan. All strains constitutively produced PG-degrading enzymes. The corresponding activity of *P. bryantii* was more than 40-fold higher than in other strains. Zymogram analyses routinely detected the presence of high-molecular-weight (100–170 kDa) polysaccharide-degrading enzymes in ruminal *Prevotella*. Characteristics of the polysaccharide-degrading activities showed diversity of ruminal *Prevotella* species.

Prevotella species are a genetically diverse bacterial group [3, 14] found in the digestive tract of ruminants, pigs, and humans [5, 13, 19]. *P. ruminicola*, *P. bryantii*, *P. albensis*, and *P. brevis* species [4], which have been earlier classified as *P. ruminicola*, are strictly anaerobic, Gram-negative pleomorphic rods or coccobacilli, and are often found in the rumen of animals fed a variety of diets. These are predominant species isolated from the rumen contents [21] and may constitute up to 60% of total bacterial isolates on a silage diet [24]. Ruminal *Prevotella* species are considered to be involved in starch digestion and utilization [6], hemicellulose and pectin digestion [7, 15], and protein or peptide metabolism [2, 25] in the rumen. For some strains of ruminal *Prevotella*, synergistic interactions with cellulolytic bacteria were observed in hemicellulose and pectin degradation in vitro [7]. Among ruminal prevotellas, *P. bryantii* strain B_14 has been intensively studied for production of endoglucanases, xylanases [9, 18], and glycosidases [11]. These enzymes were cloned, sequenced, and characterized [10–12, 16, 17, 27]. Although carboxymethylcellulase (CMCase) and xylanase activities of *Prevotella* species were detected by plating technique [4], detailed information on production and regulation of polysaccharide-degrading enzymes are limited except for the *P. bryantii* B_14 strain. Thus, the role and significance of other *Prevotella* species in polysaccharide degradation remain obscure.

In this study, we characterized polysaccharide-degrading enzyme activities of type strains of *P. ruminicola*, *P. bryantii*, *P. albensis*, and *P. brevis* grown on five different growth substrates. And also, molecular weight of each polysaccharide-degrading enzyme were estimated by zymograms.

Materials and Methods

Strains. *Prevotella ruminicola* JCM8958^T (=23^T) and *P. brevis* ATCC19188^T (=GA33^T) were obtained from Japan Collection of

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Table 1. Growth of ruminal *Prevotella* on five different carbohydrates

Species	Growth substrate				
	Xylan	Xylose	Arabinose	Cellobiose	Glucose
P. ruminicola	0.352[a]	0.080	0.410	0.613	0.496
P. bryantii	0.430	0.220	0.494	0.612	0.632
P. albensis	0.160	0.133	0.414	0.373	0.384
P. brevis	−[b]	–	0.309	–	0.122

[a] OD at 660 nm.
[b] No growth on substrate.

Microorganisms (RIKEN, Wako, Japan) and American Type Culture Collection, respectively. *P. bryantii* B_14^T and *P. albensis* $M384^T$ were kindly provided by H. J. Flint (Rowett Research Institute, UK).

Media and culture conditions. Rumen fluid cellobiose agar medium [1] was used throughout this study with minor modifications as described below. Concentration of rumen fluid was reduced to 20% (vol/vol), and 0.2% (wt/vol) of oat spelt xylan, xylose, arabinose, cellobiose, or glucose was used as sole growth substrate instead of a combination of glucose and cellobiose. Agar was omitted from the medium. The media (10 ml) were prepared anaerobically and dispensed in Hungate-type tubes (Bellco Glass, Inc.) under 100% CO_2. Cultures for inoculation were pre-grown on the same media. The inoculum (0.2 ml) was taken from the mid-exponential phase of growth. Cultures were incubated at 39°C anaerobically. Growth was monitored by optical density at 660 nm.

Enzyme assays. Cultures were incubated till mid- to late-log phase (Table 1), and the cells were harvested by centrifugation (9000 *g*, 4°C, 20 min). The resulting pellets were washed twice with ice-cold 50 mM sodium phosphate buffer (pH 6.8) and resuspended in 5% of the original volume in the same buffer. The cell suspensions were subsequently sonicated (Branson sonifier, model 250) with output 4.0, five times for 45 s, on ice. These crude cell extracts were used throughout the experiments without centrifugation. Soluble fraction of oat spelt xylan was prepared as described by Smith and Forsberg [20]. Carboxymethylcellulose (CMC), soluble xylan, and polygalacturonate (PG) were dissolved in 50 mM sodium phosphate buffer (pH 6.8) at 0.2% (wt/vol). Equal volumes of crude cell extract and substrate were mixed and incubated at 39°C. The release of reducing sugars from a substrate was monitored as previously described [23]. Glucose, xylose, and galacturonic acid were used as standards for the determination of the released monomer concentrations. Protein concentration was assayed with the Protein Assay Kit (Bio-Rad) with bovine γ-globulin as a standard. All assays were performed in triplicate. The data were statistically analyzed by ANOVA procedure.

Zymograms. Crude cell extracts (20 μl) were mixed with an equal volume of sodium dodecyl sulfate (SDS) loading buffer and denatured at 95°C for 5 min. The samples were loaded onto SDS–7.5% polyacrylamide gels containing 0.15% (wt/vol) of a substrate (described above), and electrophoresed at 150 V. After electrophoresis, the gels were renatured and incubated in 50 mM sodium phosphate buffer (pH 6.8) for 20 h at 39°C with gentle shaking. The clearing zones corresponding to enzyme activities were visualized with 0.3% (wt/vol) Congo red (stained for 10 min and destained with 1 M NaCl solution). The gel images were then captured and digitized with Fluor-S™ MultiImager (Bio-Rad) equipped with Power Macintosh. Apparent molecular masses of active protein bands were estimated with Multi-Analyst® (ver. 1.0.2) for Macintosh.

Results

P. brevis did not grow on xylan, xylose, or cellobiose medium (Table 1). All strains showed decreased growth on xylose compared with the other substrates (Table 1).

In *P. ruminicola*, the highest CMCase activities were detected in cellobiose- and glucose-grown cells (Table 2). The lowest CMCase activity was observed with arabinose-grown cells. On zymograms, the active protein bands with molecular mass of 159 kDa were commonly detected in cells grown on all substrates tested (Fig. 1-a-I). The other three active bands (102, 73, and 42 kDa) were detected in *P. ruminicola* cells grown on cellobiose or glucose. This suggests that cellobiose or glucose induced additional proteins with CMCase activities. This also results in overall higher CMCase activities (Table 2). The signal intensity of the 159-kDa protein on zymogram for arabinose-grown culture was also the weakest among substrates used, suggesting that the enzyme was repressed by arabinose.

Xylose-grown *P. bryantii* had the highest CMCase activity followed by cellobiose, xylan, glucose, and arabinose (Table 2). This trend is consistent with the earlier findings on CMCase activities of *P. bryantii* grown on the same growth substrate [9]. Two active proteins of about 90 and 40 kDa were detected on zymograms of *P. bryantii* (Fig. 1-a-II). When *P. albensis* was grown on arabinose, cellobiose, and glucose, significantly higher ($p < 0.05$) CMCase activities were observed, while no or trace activities were found on xylan and xylose (Table 2). CMCase activities of *P. brevis* were considerably lower than of other *Prevotella* strains (Table 2). For both *P. albensis* and *P. brevis*, the release of reducing sugar from carboxymethylcellulose was observed (Table 2); however, no clearing zones could be detected on zymograms (data not shown).

The highest xylanase activities were detected with *P. ruminicola* grown on xylan, cellobiose, and glucose, while the growth on xylose and arabinose resulted in lower activities (Table 2). Broad clearing zones in the range of 60–170 kDa were observed on zymograms of cellobiose- and glucose-grown *P. ruminicola* cells (Fig. 1-b-I). Positions of some xylanase bands were overlapping with that on CMC zymograms (Fig. 1-a-I, lanes 4 and 5), and this may be the enzymes expressing both xylanase and CMCase activities. In the case of *P. bryantii* and *P. albensis*, xylanase activities of the other substrate-grown cells were consistently low, but the growth on xylan resulted in significant 11- to 18-fold increase of xylanase activities ($p < 0.05$, Table 2).

Table 2. Effect of growth substrates on carboxymethylcellulase, xylanase, and polygalacturonidase activities of ruminal *Prevotella*

Enzyme activity (μmol/min/mg)	Species	Growth substrate				
		Xylan	Xylose	Arabinose	Cellobiose	Glucose
Carboxymethylcellulase	*P. ruminicola*	0.070[†a]	0.198[b]	0.018[a]	0.606[c]	0.387[d]
	P. bryantii	0.118[a]	0.302[b]	0.089[ac]	0.207[d]	0.116[ae]
	P. albensis	0.000[a]	0.015[ab]	0.365[c]	0.470[d]	0.143[e]
	P. brevis	−[‡]	−	0.008[a]	−	0.007[a]
Xylanase	*P. ruminicola*	0.667[a]	0.123[b]	0.128[b]	1.150[c]	0.889[c]
	P. bryantii	0.470[a]	0.026[b]	0.014[c]	0.019[d]	0.016[cd]
	P. albensis	0.113[a]	0.002[b]	0.007[c]	0.010[d]	0.007[ce]
	P. brevis	−	−	0.013[a]	−	0.009[a]
Polygalactoronate degrading enzyme	*P. ruminicola*	0.057[a]	0.122[b]	0.134[bc]	0.105[abd]	0.125[bcd]
	P. bryantii	7.00[a]	12.7[b]	7.03[ac]	9.13[d]	7.31[ace]
	P. albensis	0.145[a]	0.091[ab]	0.120[ac]	0.161[ad]	0.120[ace]
	P. brevis	−	−	0.150[a]	−	0.141[a]

[†] enzyme activity.
[‡] −, no growth on substrate.
[a,b,c,d,e] Values differ significantly between different superscripts within a line ($p < 0.05$).

Production of the 77-kDa xylanase of *P. bryantii* showed the strongest band intensity among growth substrates (Fig. 1-b-II). Like the CMC zymograms, no detectable xylanase activity was observed in xylan zymograms of *P. albensis* and *P. brevis* (data not shown), although the former is able to grow and display the considerable abilities to release reducing sugars from xylan. *P. brevis* possessed little activity towards xylan (Table 2) as described elsewhere [4].

All strains used in this study had PG-degrading enzyme activities. Unlike the other polysaccharide-degrading enzymes, there were no clear induction/repression effects of growth substrates on PG-degrading enzyme activity (Table 2). Production of PG-degrading enzyme (pectin-degrading enzyme) by ruminal prevotellas are constitutive among substrates used in this study. The corresponding activity in *P. bryantii* cell extracts was more than 40-fold higher than in other strains (Table 2). This suggests that this strain may play a significant role in pectin degradation in the rumen. *P. brevis* produces a single active protein band with molecular mass of 48 kDa (Fig. 1-c-IV). The other three strains produced more than two PG-degrading enzymes in the range of 40–150 kDa (Fig. 1-c-I, II, and III).

Discussion

In this study, four type strains of ruminal *Prevotella* were examined for the production and induction patterns of polysaccharide-degrading enzymes. Initial recognition of the fact that ruminal *P. ruminicola* isolates may represent a heterogeneous group has been a result of discovery of a high degree of genetic diversity in this species [3]. Further phenotypic characterization, partially based on polysaccharide-degrading profiles, divided them into the currently recognized four type species [4]. This study essentially confirms the previous findings that the 16S rDNA-based genotypic diversity has the functional meaning in terms of phenotypic (metabolic) diversity and, according to different substrate preferences, these strains may occupy distinct niches within the rumen ecosystem. However, it seems that genetic diversity of ruminal prevotellas is not limited to the current four type strains. Our molecular characterization of the rumen bacterial diversity suggested that the 16S rDNA-based diversity of ruminal *Prevotella* is well beyond this estimation [22, our unpublished data]. Isolation and phenotypic characterization of representatives of these major *Prevotella* phylogroups would identify new type strains and bring a better understanding of how the rumen functions.

Our zymogram analyses demonstrated the presence of high-molecular-weight polysaccharide-degrading enzymes in ruminal *Prevotella* (Fig. 1). The 90-kDa CM-Case band showed strong activity and is consistent with an 88-kDa endoglucanase described earlier [17], which is the biggest of β-1,4 hydrolases from ruminal prevotellas endoglucanase. Our data suggest that ruminal prevotellas possess the hydrolytic enzymes of at least double this size. These high-molecular-weight hydrolases perhaps have been missed in previous analyses because of interplay of two factors, their cellular location and the enzyme characterization methodology. At least one endoglucanase of *P. bryantii* is cell associated and cannot be detected in the supernatant during exponential growth [8]. Most cellulase purification methodologies rely on the

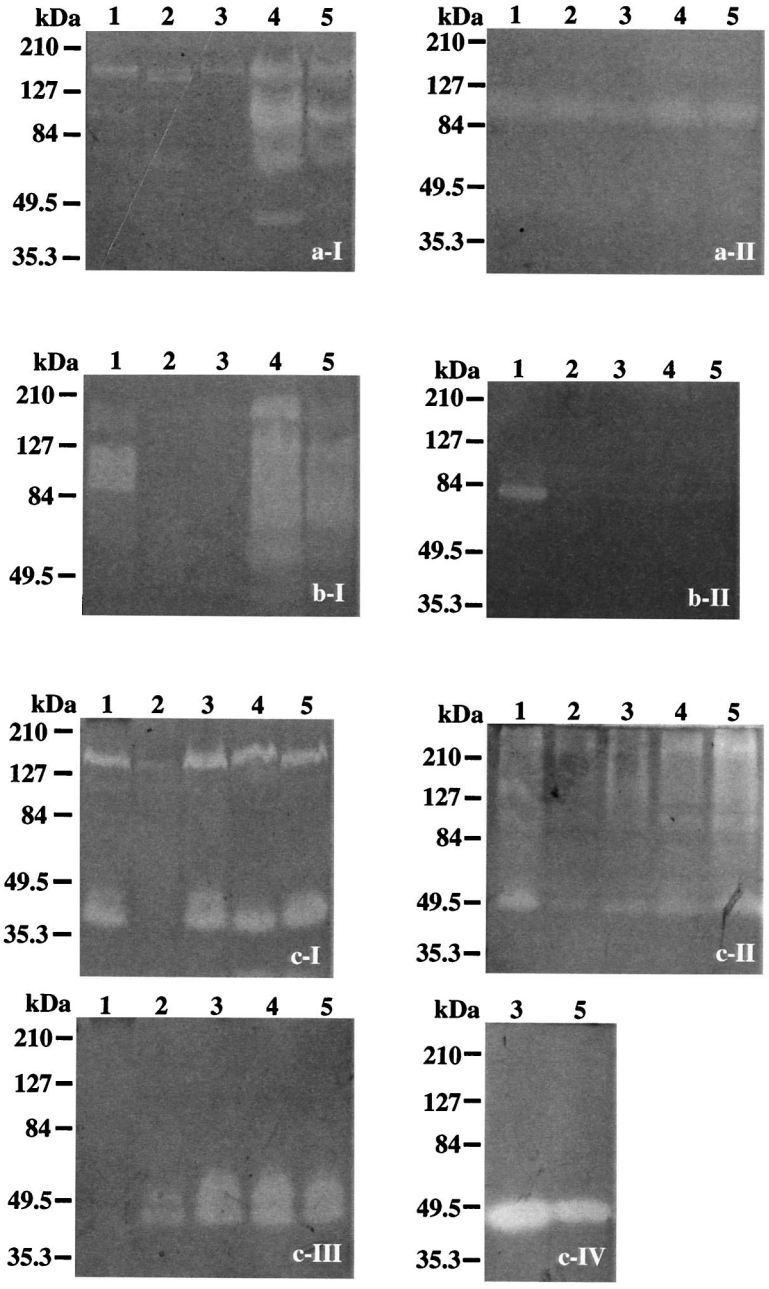

Fig. 1. Zymogram of carboxymethylcellulase (a), xylanase (b), and polygalacturonate-degrading enzyme (c) produced by four *Prevotella* type strains after grown on five different carbohydrate growth substrates. Strains: I, *Prevotella ruminicola* JCM8958^T; II, *P. bryantii* B_14^T; III, *P. albensis* M384^T; and IV, *P. brevis* ATCC19188^T. Lanes are cell extracts of *Prevotella* strains grown on xylan (1), xylose (2), arabinose (3), cellobiose (4), glucose (5).

recovery of enzymes from the supernatants. But if it is purified from the crude cell extract, then the centrifugation step is included to pellet the membrane fraction. Because of association with cell wall/membranes, these hydrolases may have escaped detection and analysis. Our protocol retained the membrane fraction for subsequent analysis, and this allowed detection of the high-molecular-weight membrane-associated hydrolases. Localization of the high-molecular-weight endoglucanase should be identified.

In *P. ruminicola*, overlapping of xylanase bands with CMCase bands was observed (Fig. 1-a-I and b-I). In fact, *P. ruminicola* 23^T (=JCM8958^T) produces a family 5 xylanase exhibiting endoglucanase activity [26] and may have other enzymes displaying mixed xylanase/endoglucanase activities.

In this study, production of polysaccharide-degrading enzymes in ruminal *Prevotella* was characterized. The growth substrates influenced production of the enzymes, and the response of each ruminal *Prevotella* was

different. Furthermore, the number and molecular masses of the enzymes were divergent. The diversity of the ruminal *Prevotella* would reflect these results.

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