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Use of Congo Red-Polysaccharide Interactions in Enumeration and Characterization of Cellulolytic Bacteria from the Bovine Rumen†

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The interaction of the direct dye Congo red with intact β-D-glucans provides the basis for a rapid and sensitive assay system for bacterial strains possessing β-(1→4),(1→3)-D-glucanohydrolase, β-(1→4)-D-glucanohydrolase, and β-(1→3)-D-glucanohydrolase activities. A close correspondence was observed between cellulolytic activity and β-(1→4)-D-glucanohydrolase and β-(1→4),(1→3)-D-glucanohydrolase activities in isolates from the bovine rumen. Many of these isolates also possessed β-(1→3)-D-glucanohydrolase activity, and this characteristic may have taxonomic significance.

The ability of microorganisms to degrade polysaccharides such as cellulose is a characteristic of considerable interest both in terms of microbial ecology and from the viewpoint of industrial microbiology. Determination of the numbers and types of cellulolytic organisms present in a complex ecosystem such as the bovine rumen, however, is a difficult problem. The use of thin agar layers containing finely divided cellulose for direct counts has been suggested to seriously underestimate the number of cellulolytic organisms present (4), although careful control of the nature of the substrate may overcome this problem (16). The alternative method of using a nonselective medium to isolate as wide a range of organisms as possible, followed by the testing of each isolate for cellulolytic activity, severely limits the nature and number of studies which can be undertaken because of the time and labor required. In both cases a degree of ambiguity arises because of the wide range in the time required for different species to produce obvious degradation of the substrate (visible degradation of intact filter paper, for example, may require as little as 2 days or longer than 2 months). Methods have been developed which overcome some of these problems, using either dye-labeled insoluble substrates (12, 14) or a soluble substrate (carboxymethyl cellulose) which is precipitated with detergent to visualize a zone of hydrolysis on an agar plate (8), but these techniques are still limited by their lack of sensitivity and by the restricted range of substrates which can be tested.

The recent demonstration that Congo red shows a strong interaction with polysaccharides containing contiguous β-(1→4)-linked D-glucopyranosyl units and a significant interaction with β-(1→3)-D-glucans and possibly some hemicellulosic galactoglucomannans (17–19) provides a new basis for the assay of β-D-glucanase activities, using unmodified soluble substrates in an agar gel plate diffusion system (P. J. Wood, Carbohydr. Res., in press). The potential advantages of this system in enumerating and characterizing cellulolytic microorganisms derive largely from the intense color of the dye-glucan complex, which allows the use of very low substrate concentrations and a corresponding decrease in the time required to detect lower levels of enzyme activity. In addition, the system allows the use of a variety of well-characterized soluble β-D-glucans to differentiate organisms on the basis of the linkages which they are able to degrade.

MATERIALS AND METHODS

Media and growth conditions. The anaerobic techniques used were based on those of Hungate (11) as modified by Bryant and Burkey (3). For plate assays an anaerobic hood (1) was employed, using an atmosphere of 80% N_2–10% CO_2–10% H_2. Media were modified for use in the anaerobic hood by reduction of the normal Na_2CO_3 level to 0.0375% (wt/vol). Medium 98-5 (5), modified by the inclusion of 0.05% xylan (9), or the medium of Mann (13) containing 2% agar and 0.2% ball-milled Whatman no. 1 filter paper was used in roll bottles for initial isolation of rumen bacteria. Isolates were characterized and identified as previous-

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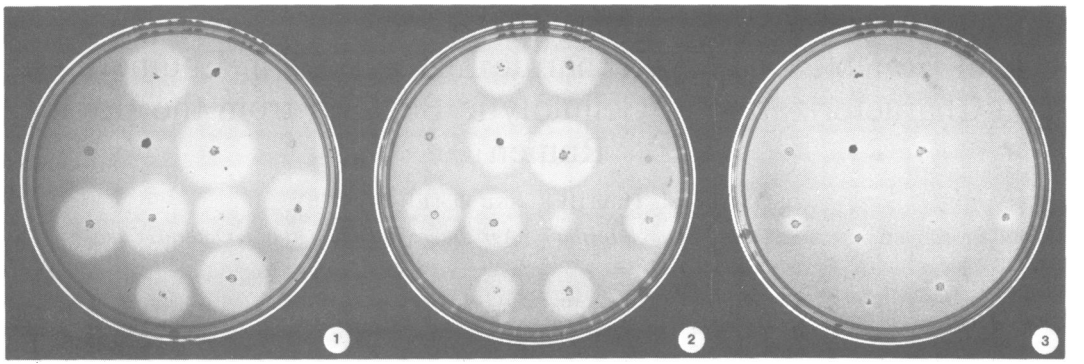

FIG. 1. Replica plates of bacterial colonies grown on medium 10 containing 0.1% cellobiose as the sole carbon source for 24 h before being overlaid with 4 ml of soft agar containing a 1-mg/ml concentration of (1) CMC, (2) QOG, and (3) CMP. The overlaid plates were incubated for a further 16 h at 37°C before being stained with Congo red. Bacterial strains are (numbering left to right and top to bottom): (1), (5), (7–12) *B. fibrisolvens*, cellulolytic isolates; (2, 4) an unidentified gram-negative nonmotile ovoid bacterium, noncellulolytic [note apparent specificity for β-(1→4),(1→3)-D-glucan]; (3) *Bacteroides* spp.; (6) *Selenomonas ruminantium*.

ly described (15) and by determination of fermentation products by gas-liquid chromatography (10).

The ability to degrade soluble β-D-glucans was tested either by inclusion of the substrate (0.05 to 0.1%) in the roll bottle medium or by overlaying colonies grown on petri plates in the anaerobic hood (medium 10 [6], modified as previously described [15]) with 4 ml of the same medium without the normal carbohydrates and containing 0.8% agar and 0.05 to 0.1% of the test substrate. The substrates used were oat β-D-glucan [QOG; β-(1→4),(1→3)-D-glucan] (19), carboxymethyl pachyman [CMP; β-(1→3)-D-glucan, prepared as described by Clarke and Stone (7)], and carboxymethyl cellulose 7H3 SXF [CMC; β-(1→4)-D-glucan, obtained from Hercules Inc., Wilmington, Del.].

Visualization of β-D-glucan hydrolysis. After an appropriate incubation period at 37°C, the agar medium was flooded with an aqueous solution of Congo red (1 mg/ml for media containing CMC or CMP; 0.2 mg/ml for media containing QOG) for 15 min. The Congo red solution was then poured off, and plates containing CMC or CMP were further treated by flooding with 1 M NaCl for 15 min. The visualized zones of hydrolysis could be stabilized for at least 2 weeks by flooding the agar with 1 M HCl, which changes the dye color to blue and inhibits further enzyme activity.

Bacterial strains. In addition to newly isolated rumen bacteria, the following stock strains were tested: *Butyrivibrio fibrisolvens* ATCC 19171, OR35, OR36, OR37, and OR38; *Clostridium cellobioparum* ATCC 15832; *Ruminococcus flavefaciens* B346 (obtained from B. A. Dehority), C94 (obtained from M. P. Bryant), and OR18; *Bacteroides succinogenes* S-85 (obtained from C. W. Forsberg); and *Eubacterium cellulosolvens* isolates 1 and 2 (obtained from N. O. van Gylswyk).

RESULTS

Initial tests using stock strains of cellulolytic bacteria showed that hydrolysis of CMC, CMP, and QOG was very rapid compared with hydrolysis of cellulose. For colonies grown on plates in the anaerobic hood (24 to 48 h) and then overlaid with 0.8% agar containing 0.1% substrate, 16 h of incubation at 37°C was sufficient to produce zones of clearing 1 to 3 cm in diameter around the colony (Fig. 1). Problems were sometimes encountered when using the overlay technique due to secondary growth in the overlay or to the presence of moisture on the plate, resulting in widespread zones of digestion. The use of well-dried plates is essential. When highly mobile or fast-growing strains are tested, the incorporation of antibiotics (100 μg of tetracycline, ampicillin, and streptomycin per ml) in the overlay is effective in preventing outgrowth. Alternatively, outgrowth of obligate anaerobes can be prevented by pouring the overlay and performing the subsequent incubation aerobically. Aerobic incubation has little effect on the apparent enzyme activity as determined by the plate overlay method.

All cellulolytic bacteria examined produced zones of hydrolysis on CMC and QOG (Table 1). Whereas CMP was in most cases degraded weakly (1 to 2-mm zone surrounding the colony, with an indistinct margin) or not at all, some strains, particularly isolates of *R. flavefaciens*, showed high activity against all three substrates (diameter of zone of clearing greater than 1.5 cm when tested by the plate overlay method). There was no apparent correspondence between the size of the zone of clearing produced on CMC or QOG and the rate at which insoluble cellulose (filter paper) was degraded. Isolates of *B. fibrisolvens* which required 21 to 71 days to produce visible degradation of intact filter paper were indistinguishable in the plate overlay assay with CMC or QOG from isolates of *R. albus* which

TABLE 1. Characteristics of cellulolytic rumen bacteria[a]

Organism	No. of isolates examined	% of isolates degrading:[b]			Days required to degrade cellulose[c]
		CMC	CMP	QOG	
R. albus	12	100 (10–20)	0	100 (5–20)	2–8 (5)
R. flavefaciens	5	100 (10–20)	100 (15–20)	100 (10–20)	1–7 (4)
B. succinogenes	4	100 (5–20)	50 (1–10)	100 (5–20)	4–5 (5)
B. fibrisolvens	21	100 (10–20)	58 (1–15)	100 (10–20)	21–71 (36)
E. cellulosolvens	17	100 (5–15)	0	100 (5–10)	2–8 (5)
C. cellobioparum	1	100 (5)	100 (3)	100 (5)	21

[a] The bacterial strains listed include the stock strains listed in Materials and Methods. Other strains were isolated from cellulose roll bottles and identified as described.

[b] Strains were tested by the overlay technique as described in the legend for Fig. 1. All tests were repeated at least twice. Numbers in parentheses give zone diameter in millimeters.

[c] Determined by using a 6-mm disk of Whatman no. 1 filter paper in 5 ml of the medium of Mann (13). The time listed is the time required to produce obvious disintegration of the filter paper. Numbers in parentheses are averages.

degraded intact filter paper in 1 to 8 days (Fig. 1).

When 100 strains were initially selected for their ability to degrade CMC and QOG, 85% were found on subsequent testing to be capable of degrading intact filter paper within 90 days. Of the remainder, most (11 of 15) showed some indication of attack on the substrate but failed to produce extensive degradation.

Viable counts of organisms capable of degrading CMC, QOG, CMP, or cellulose were determined directly from rumen fluid samples, using roll bottles containing appropriate media. The counts (zones of clearing after staining) on CMC and QOG after 2 days of incubation were similar (4×10^8 to 5×10^8 per ml of rumen fluid) and corresponded to the number of cellulolytics determined by testing randomly selected clones isolated in medium 98-5 for cellulolytic activity. Direct counts of cellulolytic organisms in the roll bottles containing ball-milled filter paper after 2 weeks of incubation (3×10^7 to 4×10^7/ml) were approximately 10% of the counts on CMC and QOG or the counts determined indirectly on strains isolated on medium 98-5. Direct counts of organisms capable of degrading CMP (1×10^7 to 3×10^7/ml) were generally considerably lower than counts on CMC or QOG, and the weak hydrolysis exhibited by many colonies made direct counts uncertain. The total viable count for these samples on medium 98-5 was 2×10^9 to 4×10^9/ml.

Table 1 summarizes the characteristics of a number of cellulolytic rumen bacteria. The range of sizes given for the zone of degradation represents strain-to-strain variation. Replicate assays were reproducible (±20%) for any specific strain. The ability to degrade the β-(1→3)-D-glucan CMP was apparently absent from strains of *R. albus* and *E. cellulosolvens*, whereas all strains of *R. flavefaciens* examined had this capability.

DISCUSSION

The use of Congo red as an indicator for β-D-glucan degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Incubation times are reduced from weeks or months to less than 1 day, and results are generally unambiguous. The method is readily adaptable to either direct counts of viable cellulolytic organisms, using roll bottles, or to the screening or characterization (or both) of large numbers of isolates, using replica plating techniques.

There is a strong correlation between the ability of bacterial isolates from the bovine rumen to degrade soluble β-(1→4) and β-(1→4),(1→3)-D-glucans (CMC and QOG) and their ability to degrade cellulose. The ability to degrade β-(1→3)-D-glucans (CMP) is also widespread among some species of cellulolytic rumen bacteria. The ecological significance of this activity is not very obvious, since β-(1→3)-D-glucans are not usually major components of plant materials. It is possible that β-(1→3)-D-glucans may restrict enzyme access to cellulosic materials, in which case production of a β-(1→3)-D-glucanohydrolase would confer a distinct competitive advantage. It is also possible that production of a β-(1→3)-D-glucanohydro-

lase may provide a competitive advantage in the rumen environment with respect to some of the cellulolytic fungi present (2).

On the basis of the limited data available, it appears that β-(1→3)-D-glucanohydrolase production may be of taxonomic significance, in that no isolates of *R. albus* (12 isolates examined) or of *E. cellulosolvens* (17 isolates examined) were able to degrade CMP, whereas all isolates of *R. flavefaciens* examined had this ability. Other species were variable in this characteristic. It should be noted that *B. succinogenes* S-85, the type strain for this species, was very active in the degradation of CMP (zone diameter, 1.2 to 1.4 cm in the plate overlay assay).

The nature of the tests applied in this study precludes any quantitative comparisons of enzyme activities. Variations in growth rate between strains and species in the media used and, in the case of the plate overlay technique, possible variations in the rate of enzyme production and diffusion during the growth period before the application of the substrate-containing overlay render any direct comparisons of activity meaningless. Nevertheless, zone diameters were qualitatively reproducible with the plate overlay technique. More quantitative results can be obtained by using liquid samples placed in wells in an agar plate containing the substrate. With this method there is a direct relation between log enzyme concentration and the diameter of the cleared zone, and the sensitivity of the assay system offers considerable promise for future studies on rumen cellulases. For example, centrifuged rumen fluid generally shows detectable β-(1→4)-D-glucanohydrolase activity at dilutions down to at least 1/32, and variations between animals and diets are readily apparent.

All cellulolytic rumen bacteria examined in this study produced zones of digestion of CMC and QOG in agar media, indicating that in all cases a diffusible β-(1→4)-D-glucanohydrolase, at least, was produced. Thus, whereas both cellulolytic bacteria and cellulolytic enzymes are normally closely associated with their substrates, the association between bacteria and enzyme is not necessarily a direct one. It should be noted, however, that in this assay the bacterial colonies assayed would be expected to contain largely stationary-phase cells, and enzymes which are normally closely associated with the cell might be released through cell lysis.

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