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Improved Assay for Quantitating Adherence of Ruminal Bacteria to Cellulose

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A quantitative technique suitable for the determination of adherence of ruminal bacteria to cellulose was developed. This technique employs adherence of cells to cellulose disks and alleviates the problem of nonspecific cell entrapment within cellulose particles. By using this technique, it was demonstrated that the adherence of *Ruminococcus flavefaciens* FD1 to cellulose was inhibited by formaldehyde, methylcellulose, and carboxymethyl cellulose. Adherence was unaffected by acid hydrolysates of methylcellulose, glucose, and cellobiose.

The adherence of gut microbes to plant digesta was observed in early studies of ruminal contents by light microscopy. Microscopic observations of bacteria within lacunae, i.e., zones of digestion, led early workers to suggest that adherence was an important factor in plant degradation (2, 3). More recently, the adherence of ruminal microbes to plant material has become the focus of renewed research interest (1, 11, 12), but progress has been hampered by a lack of reliable quantitative techniques for studying the adherence process. Therefore, we developed a technique to measure bacterial adherence to cellulose that is suitable for quantitative research.

Ruminococcus flavefaciens FD1 and C94, *Bacteroides succinogenes* S85, *Ruminococcus albus* 7, and *Butyrivibrio fibrisolvens* A38 and 49 were obtained from the culture collection of M. P. Bryant, Department of Animal Sciences, University of Illinois, Urbana. *B. fibrisolvens* 12 and X6C61 and *Clostridium polysaccharolyticum* B were obtained from N. O. van Gylswyk, Council for Scientific and Industrial Research, National Chemical Research Laboratory, Pretoria, Republic of South Africa. *Eubacterium cellulosolvens* 5494 was obtained from W. E. C. Moore, Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg.

Preliminary adherence experiments were conducted with cellulose particles by the turbidometric methods of Minato and Suto (13). All experiments were conducted at 39°C for 30 min, and the decrease in optical density after cellulose sedimentation was used as an indicator of microbial cell adherence to cellulose.

Preliminary adherence assays demonstrated that several species of ruminal bacteria were capable of associating with cellulose particles (Table 1), in agreement with previous studies (13, 15). Noncellulolytic or weakly cellulolytic strains adhered to a lesser extent than did the actively cellulolytic strains. Adherence to pebble-milled cellulose was greater than the adherence observed with the more crystalline cellulose (Sigma Chemical Co.). Presumably, the finer particle distribution of pebble-milled cellulose (8% no. 1 filter paper [Whatman, Inc.] in water, milled 72 h) provided more sites for adherence, as suggested by Hobson (5). However, greater physical entrapment of cells cannot be excluded as an equally important factor.

Adherence assays using finely divided cellulose, such as those used in these preliminary experiments and as reported by other workers (13–15), were judged to be inadequate for a number of reasons, and alternative methods were investigated. It was demonstrated in our preliminary experiments that a method using cellulose particles and sedimentation was unacceptable especially with chain-forming microbes such as *R. flavefaciens* FD1. These microbes were easily entrapped in the cellulose suspension, and in fact, with strain FD1, cells were sedimented at low-speed centrifugation even when no cellulose was present. Such entrapment was quite variable, making it impossible to distinguish entrapment from true adherence, even when a variety of experimental methods, including differential centrifugations and washings, were used.

An improved adherence assay was developed to determine the adherence of ^{14}C-labeled *R. flavefaciens* FD1 to acid-swollen cellulose disks. Cellulose-grown cultures (0.15% [wt/vol] acid-swollen cellulose) were specifically labeled with 150 nmol of [1-^{14}C]2-methylbutyrate per ml (0.025 μCi of [1-^{14}C]2-methylbutyric acid-sodium salt per ml; California Bionuclear Corp.). The basal medium and acid-swollen cellulose disks used were as previously described (16). Typically, under these growth conditions, 15% of the added radioactivity was incorporated into bacterial cells. Although these levels were low and could not be increased by varying the amounts of 2-methylbutyrate used, the incorporated label was found mainly in lipid-containing materials and not in cellular or excreted proteins. Protein content was determined by the method of Bradford as previously described (16).

Labeled cells were used in the following adherence assay. A sample of cell cultures (0.1 ml; optical density at 600 nm, 0.9) was added to reduced, warmed basal medium (1 ml) which contained 10 acid-swollen cellulose disks (28.0 ± 0.5 mg [dry weight]) and, where appropriate, potential inhibitors. Both methylcellulose (MC; 15 and 400 cP) and carboxymethyl cellulose were obtained from Sigma and washed prior to use as previously described (16). After inoculation, the assay tubes were stoppered anaerobically and placed horizontally on a reciprocating shaker (39°C). This arrangement afforded gentle agitation and adequate opportunity for adherence. Sample tubes were collected at specific time intervals (optimally at 45 min) and immediately processed.

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TABLE 1. Adherence of ruminal bacterial species to cellulose

Bacterium	% Adherence to cellulose type[a]	
	Crystalline	Pebble-milled[b]
E. cellulosolvens 5494	77	107
Bacteroides succinogenes S85	56	120
R. albus 7	80	101
R. flavefaciens C94	7	88
C. polysaccharolyticum B	10	65
B. fibrisolvens A38	78	108
B. fibrisolvens 12	13	26
B. fibrisolvens 49	18	33
B. fibrisolvens X6C61	3	29

[a] Determined by the turbidometric method.
[b] Values exceeding 100% are experimental artifacts due to calculating percent adherence by difference (13).

The liquid medium was removed, and the cellulose disks were washed three times with 1 ml of water each time by gentle inversion of the tube. Control experiments indicated that water and basal medium were equally effective wash solutions. After the washing, the cellulose disks were transferred to scintillation vials, 5 ml of Aquasol II (Du Pont Co.) was added to each vial, and the samples were counted for 20 min by liquid scintillation counting. All counts were corrected for quenching by using a standard curve. Each treatment was conducted in triplicate, and the results of the assay were found to be linear with cell protein concentration (Fig. 1A) and with time (Fig. 1B).

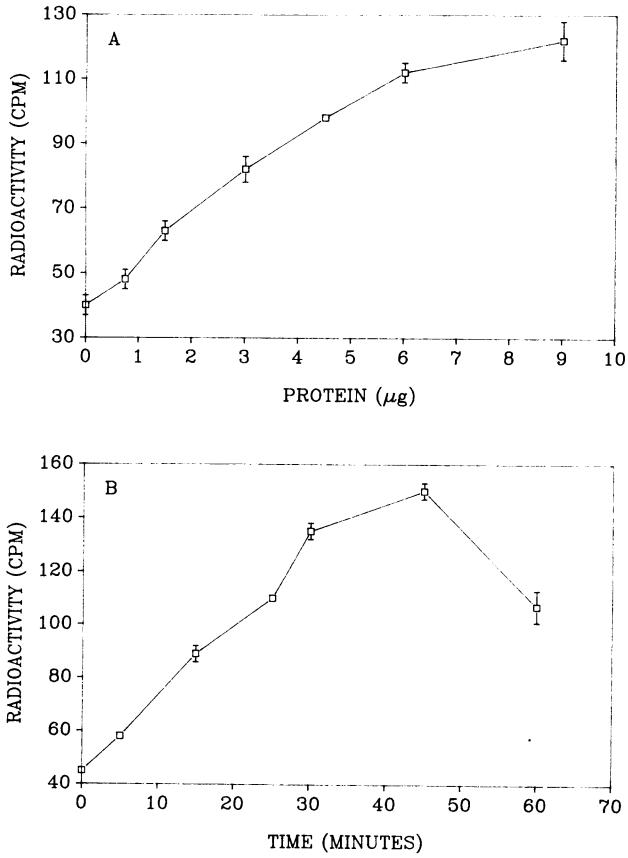

FIG. 1. Adherence of ^{14}C-labeled cells of *R. flavefaciens* FD1 to cellulose disks. Linearity test of assay with respect to cell protein (45-min incubation time) (A) and to incubation time (B).

The adherence of ^{14}C-labeled bacterial cells to standardized cellulose disks was found to be a more reliable assay. By using this assay, it was demonstrated that the adherence of *R. flavefaciens* FD1 was inhibited by 10% (vol/vol) formaldehyde. This observation contradicts the data of Minato and Suto (13). They used a turbidometric adherence assay and reported no effect with such a treatment. We believe that these conflicting data reinforce the questionable value of turbidometric assays employing cellulose particles. In turbidometric assays, cells fixed with formaldehyde could have been entrapped in the sedimented cellulose. Our data are also consistent with the concept that adherence to cellulose is a protein-mediated event, as has been previously suggested (4, 7, 9, 11).

Adherence was unaffected by the presence of soluble carbohydrates (cellobiose or glucose) in the assay mixture (Table 2) or by changes in pH (range, 6.0 to 8.0; data not shown). The insensitivity of *R. flavefaciens* adherence to changes in pH is consistent with other data for the adherence of *R. albus* to cellulose (14). However, these data disagree with adherence data for a mixed population of rumen microorganisms (6), which most likely contain multiple species of cellulolytic microorganisms.

Adherence was also unaffected by addition of low-molecular-weight MC acid hydrolysates. In contrast, adherence was inhibited by highly viscous MC (400 cP) (Fig. 2) and by carboxymethyl cellulose. In light of previous evidence that MC inhibits glucanases responsible for cellulolysis (18), adherence by *R. flavefaciens* may be an enzyme-substrate-mediated process, as has been previously suggested for cellulolytic microbes (7, 8, 13, 14, 17). There was a lesser degree of inhibition of adherence by the low-viscosity MC (15 cP), and these data are consistent with those reported for *Clostridium thermocellum* (10). It is possible that high-molecular-weight cellulose derivatives (MC, 400 cP, average M_w = 41,000 versus MC, 15 cP, average M_w = 15,000) display significant differences such that only the higher-

TABLE 2. Effects of additions on adherence of *R. flavefaciens* FD1 to cellulose disks

Addition (concn)	Degree of adherence			
	Expt 1		Expt 2	
	cpm[a]	%	cpm[a]	%
None	121 ± 14	100	87 ± 8	100
Formaldehyde (10% [vol/vol])	31 ± 2	26	14 ± 3	16
Sodium arsenate (10 mM)	100 ± 6	83	ND[b]	
Cellobiose (10 mM)	111 ± 5	92	83 ± 8	95
Glucose (10 mM)	110 ± 4	91	ND	
MC[c] hydrolysate (DP, 9.5)[d] (10 mM)	132 ± 13	109	92 ± 15	106
MC[c] hydrolysate (DP, 4.5) (10 mM)	112 ± 8	93	101 ± 7	116
MC[c] hydrolysate (DP, 1.0) (10 mM)	114 ± 11	94	91 ± 8	104
Carboxymethyl cellulose (0.1% [wt/vol])	38 ± 4	31	33 ± 2	38
MC, 400 cp (0.1% [wt/vol])	18 ± 3	15	28 ± 2	32
MC, 15 cp (0.1% [wt/vol])	75 ± 15	62	63 ± 5	72

[a] Means ± standard deviations, standardized to 6 μg of total culture protein added.
[b] ND, Not determined.
[c] Glucose equivalent.
[d] DP, Degree of polymerization.

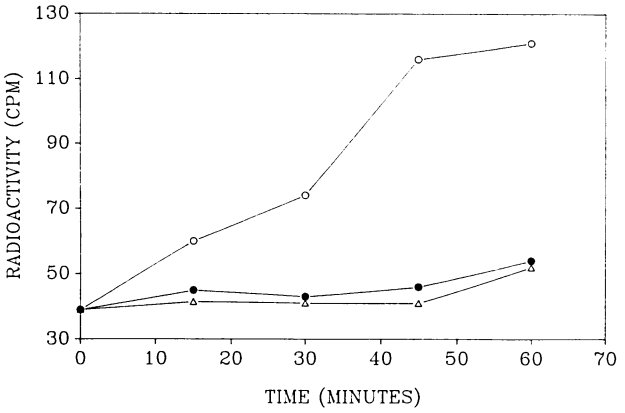

FIG. 2. Adherence of ^{14}C-labeled cells of *R. flavefaciens* FD1 to cellulose disks (MC, 400 cP). Symbols: ○, no MC added; ●, 0.02% (wt/vol) MC; △, 0.05% (wt/vol) MC.

molecular-weight preparation is inhibitory. One possible explanation may be that the adherence receptor for *R. flavefaciens* resides at a site distant from the cellulose hydrolysis site, and only the larger cellulose derivatives are capable of blocking both sites.

In summary, a technically simple, quantitative adherence assay was developed. With slight modifications, this assay can be used to measure adherence of a variety of other ruminal and nonruminal microorganisms.

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