Selective Isolation and Characterization of Cellulolytic Bacteria by Cellulose Enrichment Method from the Rumen of Ruminants

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Abstract

A simple method using medium enriched with Whatman cellulose filter paper as the sole selective substrate, was very effective in the isolation of cellulolytic bacteria from the rumen of ruminants. The rumen samples were incubated in enrichment broth medium for 36 to 96 hours prior to inoculation into non-selective glucose-cellobiose agar

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roll tubes. Of the 120 colonies picked from these roll tubes in Canadian study, 45 were found to be cellulolytic. The 36 out of 90 colonies obtained from the water buffalo samples in the Malaysian study were cellulolytic. Similarly, 260 out of 420 colonies picked in the Japanese study were cellulolytic. The 10 out of 16 colonies in the Argentinian study were cellulolytic. Most of these cellulolytic bacteria were identified as *Fibrobacter succinogenes*. *Ruminococcus flavefaciens* was found less frequently, and no *Ruminococcus albus* was isolated. None of the colonies were contaminated with cells of *Treponema* species, but contamination by *Butyrivibrio* species was noted. The method described in this paper is effective, requires less time than the conventional cellulose agar method or soluble carbohydrates-agar medium, and is superior to the latters because pure colonies of *F.succinogenes* are detected as being cellulolytic in broth cellulose medium whereas they escape detection by conventional cellulose agar method because they fail to produce clear zones.

Additional key words: *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens*, *Treponema*, cattle, water buffalo.

Introduction

The rumen contains a highly complex community of large number of microorganisms including species which are capable of digesting cellulose. Cellulose digesting microorganisms are very important for the degradation of plant materials in ruminants because they are involved in the initial attack of cellulosic materials, and provide monomeric products as substrates to other microorganisms which can not directly degrade this polymer.

When grass leaves that had been exposed to colonization and digestion by rumen bacteria were examined by transmission electron microscopy[1, 2, 3, 4] large areas of the surface of these plant cell walls appeared to be colonized by pleomorphic cells of *Fibrobacter succinogenes*[9]. Cheng *et al.*[9] reported that when straw is exposed to natural populations of rumen bacteria, the thick highly structured cellulosic cell walls of this plant material are colonized predominantly by a Gram-negative rod-shaped bacterium resembling *F. succinogenes*. A less common colonizing organism on straw is a thin-walled Gram-positive coccus resembling *Ruminococcus flavefaciens*. Attempt to isolate *F. succinogenes* from the rumen and to maintain a strong cellulolytic activity under laboratory conditions have not always been successful[25, 26] These difficulties have limited the number of strains of *F. succinogenes* used *in vitro* digestion studies to only a few strains[7, 8, 11, 12, 13, 14, 19, 23, 25, 26]. Therefore, it is important to isolate additional strains of this bacterium that will maintain a cellulolytic activity in pure culture, in order to gain a better understanding of the role of *F. succinogenes* in the rumen cellulose digestion. In this paper, we describe a simplified enrichment method, with cellulose paper as the selective substrate, and illustrate its usefulness for the isolation of *F. succinogenes* from the rumen of ruminants.

Materials and Methods

Rumen content inoculum

The solid rumen contents and rumen fluid used were collected from fistulated Holstein cattle in Canada, Japan and Argentina, and a fistulated water buffalo in Malaysia, 2 hours after the morning feedings. The cattle were fed daily amounts of hay cubes equivalent to 0.5% of the body weight, with chopped straw offered as free choice. The water buffaloes were fed molasses

equivalent to 0.1% of the body weight plus mineral supplements and palm press fiber *ad libitum* daily. One part of the rumen fluid and one part of the solid contents (preferably taken from the upper third of rumen contents were blended for 30 seconds at low speed, allowed to stand for 30 seconds, blended for 30 seconds at high speed, squeezed through two layers of cheese cloth or gauze and used as inocula.

Cultue techniques and media

All the media were prepared under strictly anaerobic conditions with a CO_2 gas phase, by applying essentially the technique of Hungate[16] modified by Bryant and Burkey[6]. The liquid enrichment medium (10 mL) used for initial enrichment and maintenance of bacteria was a modification of that of Scott and Dehority[24], with 1.5cm^2 (about 20 mg) of Whatman cellulose paper as a sole source of carbohydrate (MOD-SD). In the case of *F. succinogenes*, Whatman No.5 or 6 cellulose paper (crystalline cellulose) without any soluble carbohydrate should be used while amorphous (e.g. ball-milled) materials with 0.3% cellobiose (w/v) should be used for the isolation of *Ruminococcus* in the medium for isolation. The same medium, with a reduced amount (3 mm^2) of cellulose paper, was used for the detection of cellulolytic bacteria. The dilution fluid consisted of MOD-SD medium lacking rumen fluid and carbohydrate. For roll tubes for bacterial isolation, the MOD-SD medium was supplemented with 0.025% (w/v) each of cellobiose and glucose, and 1.8% (w/v) of agar, as described by Grubb and Dehority[15]. After the isolation soluble carbohydrates (especially glucose) should never be used in the medium since they drastically reduce cellulase activities.

Isolation and characterization of cellulolytic bacteria

One milliliter of inoculum was incubated in 10 mL of the enrichment medium at 39°C for 36 to 96 hours. After incubation, the tubes were vortexed until the filter papers were completely disintegrated, then cooled to 0 °C by inserting them into ice for 30 minutes, vortexing occasionally to release cellulolytic bacteria from the cellulosic fibers[22]. One milliliter of the 10^{-7}, 10^{-8}, and 10^{-9} dilution was inoculated into glucose-cellobiose agar roll tubes, spun mechanically (Tube spinner, Bellco Glass, Inc., Vineland, N.J., U.S.A./Roll agar tube maker, Sanshin Kogyo Co., Koohoku-ku, Yokohama, Japan), and incubated at 39°C for 36 to to 96 hours. All the colonies were picked from each of the tubes of the dilution which yielded approximately 30 colonies per tube. These were inoculated into the detection medium and incubated at 39°C. Cultures from tubes of the detection medium showing the visible signs of filter-paper disintegration were transferred to enrichment medium for maintenance (Fig. 1).

Culture purity and cellular morphology were examined by phase contrast microscopy and electron microscopy, and strains were characterized by gas chromatography of fermentation products. For this characterization, cultures were grown for 5 days in Scott and

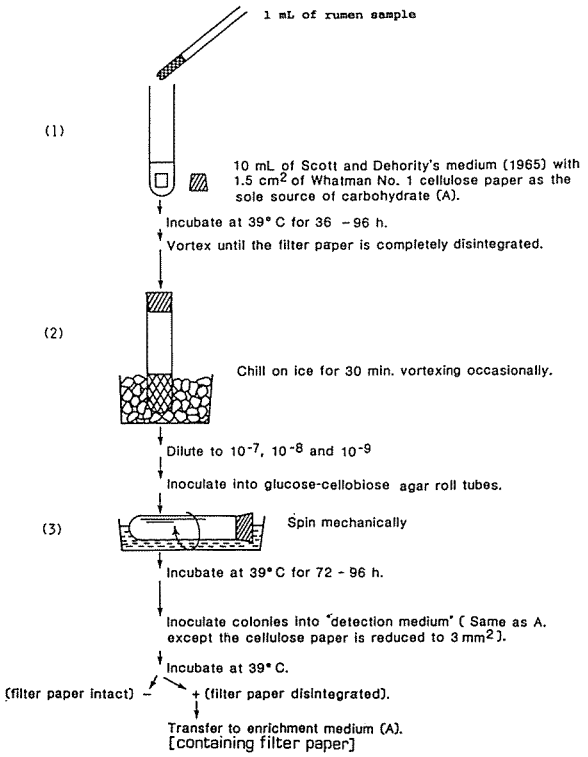

Fig. 1. Simple isolation method for rumen cellulolytic bacteria

Dehority's medium containing 30 mg of cellobiose or cellulose (as Whatman No.1 filter paper). Method for VFAs, lactic acid, succinic acid and ethanol analyses was described previously[17]. For "clear zone" formation tests, 1.3% (w/v) of enzymatically treated Whatman No.1 cellulose paper or untreated ball-milled Whatman No.1 filter paper was substituted for the carbohydrates in the medium of Scott and Dehority[24], and 1.8% (w/v) agar was added. Filter paper cellulose was enzymatically prepared by placing 12g of shredded Whatman No.1 filter paper in 1 L of culture waste from a cellulolytic bacterium for 96 hours, with stirring, following by washing and autoclaving the treated fiber.

Electron microscopy

Partially digested cellulose filter papers from 24-hour enrichment cultures were prefixed for 1 hour by the addition of a fixative solution diluted 1:10 with cacodylate buffer and containing 0.15% (w/v) ruthenium red. The cellulose fibers were allowed to settle out. For scanning electron microscopy (SEM, Hitachi 500 series), the samples were internally metallized by applying modified thiocarbohydrazide technique[20], dehydrated, critical point dried[10], and examined without metal evaporation.

Results

Direct SEM examination of filter paper from 24-hours enrichmenrt cultures of samples from the rumen of the cattle showed that the surfaces of these cellulosic materials were heavily colonized by bacteria. The characteristic spiral cells of *Treponema* species and curved rod-shaped cells resembling *Butyrivibrio* (Fig. 2) were associated with the fiber surface while coccoid and rod-shaped cells were embedded to various extents in the cellulose surface.

Culture material from enrichment culture derived from the rumen contents of the cattle in the Canadian study was used to inoculate roll tubes and 120 colonies were picked from the dilution series that yielded about 30 colonies per tubes. Of these 120 colonies, 45 colonies were found to be cellulolytic on the detection medium. Thirty-two of these 45 colonies consisted of pure strains resembling either *F. succinogenes* or *R. flavefaciens*. Thirteen colonies were composed of a mixture of cells resembling *F. succinogenes* and *Butyrivibrio* sp., *F. succinogenes* and unidentified rod-shaped bacterium. None of the colonies picked from the roll tubes contained cells resembling *Treponema* species even though direct observation of cellulose fibers from the original enrichment cultures revealed large numbers of these spiral organisms. Since the cellulolytic rods were Gram-negative and produced large amounts of acetic and succinic acids from cellulose (Table 1) we concluded that these rods corresponded to cells of *F. succinogenes*. None of the pure strains of cellulolytic rods were able to produce "clear zone" in the cellulose (Whatman No.1 cellulose paper, enzymatically

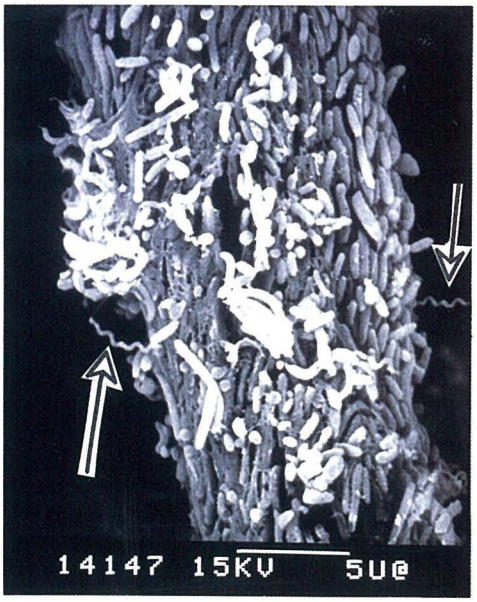

Fig. 2. Examination of partly digested filter paper fibers from 24-h enrichment cultures inoculated with rumen contents from a water buffalo

Scanning electron micrograph showed that the surface of these cellulose fibers were heavily colonized by a mixture of rod-shaped bacteria and of slightly elongated coccoid bacteria, and spiral cells (arrows) resembling those of *Treponema* sp. were associated with these primarily colonizers.

Table 1. Fermentation products generated by strains of *Fibrobacter succinogenes* isolated from the rumen of a steer in Canada

Strain	Fermentation products (mM)*		Strain	Fermentation products (mM)*	
	Acetic acid	Succinic acid		Acetic acid	Succinic acid
A	5.0	7.9	T	4.2	7.7
B	4.6	6.6	U	4.6	7.4
C	5.5	7.7	V	4.2	7.9
D	5.2	7.5	W	4.2	6.3
E	4.0	6.7	Y	3.5	7.6
F	4.5	7.2	Z	5.2	8.7
G	3.8	6.9	a	5.0	9.2
H	4.6	7.5	b	4.9	8.8
I	3.5	7.1	d	4.6	7.6
J	3.2	7.1	e	3.7	7.8
K	4.1	8.0	f	2.5	7.2
L	4.4	7.5	g	2.6	7.1
M	5.5	7.9	h	2.6	7.6
N	4.6	7.5	i	2.8	8.4
O	4.8	7.5	j	2.5	7.7
P	4.2	7.8	m	3.1	8.2
Q	3.5	6.9	n	3.1	8.7
R	3.7	7.7	o	3.9	8.7
S	3.6	7.6			

*Cultures were incubated for 5 days in 10 mL of Scott and Dehority medium with 30 mg of Whatman No. 1 cellulose filter paper.

prepared or untreated) agar roll tubes. After similar purification, the rod-shaped cells resembling *Butyrivibrio* sp. also failed to show a cellulolytic activity in the cellulose agar roll tubes. However, coculture of *F. succinogenes* with the curved, rod-shaped cells resembling *Butyrivibrio* sp could produce clear zones in both types of cellulose agar roll tubes. Fermentation products from cellobiose generated by the curved rod-shaped cells varied considerably among the isolates (Table 2). From 30 mg of cellobiose, they produced between 4.3 and 12.6 mM butyric acid and between 1 and 20 mM lactic acid. Based on their morphology and their fermentation products (Table 2), we concluded that the curved, rod-shaped cells corresponded to *Butyrivibrio* sp. Examination of 12-hour culture of the cellulolytic cocci revealed the presence of Gram-positive cells arranged in pairs or in chains of 5-14 cells. In phase contrast microscopy, the cells in older cultures (>18 hours) appeared to be spherical or elongated and they occurred predominantly as diplococci and were found to be Gram-negative or

Table 2. Fermentation products generated by strains of *Butyrivibrio* sp. isolated from the rumen of a steer in Canada

Strain	Fermentation products (mM)*	
	Butyric acid	Lactic acid
B	11.8	1.0
E	11.5	2.0
X	11.2	1.8
e	10.5	2.1
f	4.3	20.0

*Cultures were incubated for 5 days in 10 mL of Scott and Dehority medium with 30 mg of cellobiose.

Gram-variable. Major fermentation products from cellulose included acetic and lactic acids (Table 3), but one strain did not produce lactic acid. None of the strains produced ethanol. All purified strains could produce "clear zones" accompanied by yellow pigment production on both treated and untreated Whatman cellulose agar roll tubes. Yellow pigment production was also observed in the broth medium of Scott and Dehority with cellulose paper as the sole source of carbohydrate.

Table 3. Fermentation products generated by strains of Ruminococcus flavefaciens isolated from the rumen of a steer in Canada

Strain	Fermentation products (mM)*		
	Acetic acid	Lactic acid	Succinic acid
1	2.9	2.2	4.5
2	3.4	2.2	4.4
3	2.8	2.0	4.4
4	3.3	2.3	4.1
5	3.5	1.7	4.2
6	2.7	0.9	4.3
7	4.9	-	5.3

*Cultures were incubated for 5 days in 10 mL of Scott and Dehority medium containing 30 mg of Whatman No.1 cellulose filter paper. None of the strains produced ethanol.

Based on the cellular fermentation products, and the production of the characteristic yellow pigment, these cellulolytic cocci were characterized as *R. flavefaciens*.

When material from enrichment cultures derived from the rumen contents of the buffalo in the Malaysian study was used to inoculate roll tubes, 90 colonies were picked and identified. Thirty-six of these 90 isolates displayed a cellulolytic activity when transferred to the detection medium. Of the 36 cellulolytic isolates, 29 were identified as *F. succinogenes* and six were identified as *R. flavefaciens*. Only one culture of *F. succinogenes* was contaminated with *Butyrivibrio* sp. The net fermentation products (Table 4) of the *F. succinogenes* isolated from enrichment cultures

Table 4. Fermentation products generated by strains of *Fibrobacter succinogenes* isolated from the rumen of a water buffalo in Malaysia

Strain	Fermentation products (mM)*			
	Acetic acid	Butyric acid	Succinic acid	Lactic acid
101	6.9	-	11.3	-
103	5.7	-	11.8	-
104	8.1	-	11.5	-
105	6.3	-	10.5	-
107	7.0	-	11.5	-
108	6.4	-	9.4	-
109	1.9	1.0	-	-
110	2.4	-	8.7	0.7
112	10.1	1.3	11.5	-
113	3.6	-	9.8	1.8
114	8.0	-	10.2	1.7
116	7.3	0.6	12.7	1.2
117	-	-	11.8	0.7
118	-	4.5	-	0.5
119	4.5	2.1	9.7	1.0
120	4.4	-	12.3	1.8
121	7.4	1.2	10.1	0.8
122	3.9	-	10.5	0.7
123	6.1	1.2	12.6	1.1
124	4.7	0.4	0.4	0.6
125	-	3.9	10.6	1.2
126	5.6	-	12.7	1.7
127	7.1	0.6	11.9	1.2
128	4.8	-	11.1	2.8
129	6.0	-	9.4	2.1
130	3.4	-	9.0	1.7
131	3.0	-	-	1.1
133	4.3	1.5	0.2	1.7
134	1.4	0.3	0.2	1.2
135	5.6	-	9.3	0.5

*Cultures were incubated for 5 days at 39°C in 10 mL of Scott and Dehority medium with 30 mg of Whatman No.1 cellulose filter paper.

from the buffalo indicated thar the majority of them produced large amounts of succinate and acetate, and most of them produced a small amount of lactic acid. The fermentation products of a few isolates were very low which may indicate that these cultures were losing their cellulolytic activity. *F. succinogenes* isolated from the cattle could multiply and digest cellulose in media without rumen fluid whereas most strains from the water buffalo could do so only in media containing rumen fluid. In addition to the above-noted differences, there was a general similarity in morphology, fermentation products, and cellulolytic activity between the strains of the *F. succinogenes* isolated from the cattle and those isolated from the buffalo. The six strains of *R. flavefaciens* isolated from the buffalo showed a higher production of acetate and succinate from cellulose than those from the cattle, except for two strains (111 and 132) which had very low levels of succinic acid (Table 5).

Similarly, 260 out of 420 colonies picked in the Japanese study were cellulolytic (Table 6). In this study ratio of contamination was 11/260. The 208 out of 260 cellulolytic bacteria were *F.succinogenes.*

The 10 out of 16 in the Argentinian study were cellulolytic (Data not shown). All the 10 strains were *F. succinogenes.*

Direct examination, by phase contrast microscopy, of cells from pure cultures of *F. succinogenes* isolated from either the cattle or the buffalo showed that the cells of the fresh isolates consisted rods with various sizes and that continued subculture induced a uniformly smaller cell size and a higher degree of polymorphism.

Discussion

It is now generally recognized in the field of microbial ecology that many bacteria live and function with other species in operative consortia[17]. We have developed a new and very simple method of examining the process of cellulose digestion in ruminants, which emphasizes the concept of consortial bacterial activity, and we

Table 5. Fermentation products generated by strains of Ruminococcus flavefaciens isolated from the rumen of a water buffalo in Malaysia

Strain	Fermentation products (mM)*		
	Acetic acid	Lactic acid	Succinic acid
102	6.5	-	8.5
106	6.9	-	6.8
111	2.5	0.9	0.1
115	6.5	2.2	7.8
132	8.7	0.5	0.2
136	8.7	0.6	9.4

*Cultures were incubated for 5 days in 10 mL of Scott and Dehority medium containing 30 mg of Whatman No. 1 cellulose filter paper.
None of the strains produced ethanol.

Table 6. Comparison of isolation methods for cellulolytic bacteria from the rumen of cattle in Japan

	Conventional method (Soluble carbohydrate -agar roll tube)	Cellulose-enrichment method (Figure 1)
Cellulolytic bacteria /Total number of picked colonies	13/450	260/420
Ration of active cellulolytic bacteria	11/13	260/260
Ratio of contamination	3/13	11/260
Fibrobacter succinogenes /Total number of cellulolytic bacteria	1/13	208/260

have matched these cultural data with morphological data obtained by direct observation of partly digested cellulose. Direct SEM observations of enrichment cultures from the cattle and the buffalo revealed that cellulose fibers are heavily colonized by a mixed bacterial population within which rod-shaped bacteria, elongated coccoid bacteria, and spiral organisms resembling *Treponema* sp. can be recognized. Difficulty in isolating and enumerating cellulolytic bacteria has been documented[26] and is likely to have led to the underestimation of the contribution of this sector of the rumen microbial population. In particular, it has been difficult to detect and isolate *F. succinogenes* because of its inability to produce clear zones in cellulose agar. Consequently, much of our knowledge about this organism has derived from the study of only a few strains. In view of the tendency of this species to induce physiological and morphological changes after prolonged laboratory culture, it is necessary to develop a simple method by which fresh isolates may be obtained. Additionally, the study of a larger number of strains of *F. succinogenes* is essential to gain a better understanding of the morphological and physiological diversity exhibited by strains of these species (Tables 1 and 4).

Our new isolation method includes an enrichment phase which is conducive to the development of naturally occurring digestive consortia, and allows for the cellulolytic portion of the rumen population to be enhanced so that these species can still be recovered in serial dilutions suitable for colony isolation from roll tubes. The use of a specific liquid medium containing filter paper allows for the detection of cellulolytic activity in the roll tube isolates, notably also in pure culture of *F. succinogenes* and possibly of other species which previously had not been detected because of their inability to clear cellulose agar. Cellulolytic activity present in the fresh isolates is maintained by the presence of cellulose paper as the sole source of carbohydrate in the maintenance medium. Using this method, were recovered 120, 208, 10 and 90 isolates from the rumen contents of cattle in Canada, Japan and Argentina, and water buffalo in Malaysia, respectively, and detected a cellulolytic activity in approximately 40% of the isolates in all the cases. All the isolates containing *R. flavefaciens* also produced clear zones in conventional cellulose agar tubes; however, isolates of *F. succinogenes* did not produce this "clearing" unless other organisms such as *Treponema* and *Butyrivibrio* were present in coculture. This finding may account for the generally reduced recovery of *F. succinogenes* as cellulolytic organisms, in relation to *Ruminococcus* species, and for the finding that isolates of *F. succinogenes* detected through cellulose clearing assays are always contaminated by *Treponema, Butyrivibrio,* and other bacterial species[5, 16, 17].

Both the morphological and physiological data obtained in this study indicate a general similarity between the cellulolytic bacterial populations of the cattle and water buffalo. Spiral cells resembling *Treponema* species were revealed by SEM in all the animals, and *F. succinogenes*, *R. flavefaciens*, and *Butyrivibrio* sp. were all observed, isolated and characterized in both animals. However, direct examination by SEM showed cocci were more numerous in the bacterial population on cellulose fibers in enrichment cultures from the buffalo rumen compared with that from the cattle rumen. Fermentation products were generated at higher concentration by *R. flavefaciens* from the buffalo rumen, and both *F. succinogenes* and *R. flavefaciens* from the buffalo rumen showed greather dependence on rumen fluid. These minor microbial differences may result from the influence of diet[18, 19, 21, 27] on bacterial rumen populations (e.g., straw vs. palm press fiber) but they may also be related to the greater efficacy with which water buffalo can utilize low quality cellulosic feeds. However, we found negligible degradation of palm press fiber in the rumen.

We consider that for the ecological examination of complex bacterial populations, such as these cellulolytic rumen communities, morphological and isolation method should be combined so that investigators can be certain that

most of the members of important consortia are "accounted for" among the isolates. Even after isolation, we must realize that some species cannot function (e.g., clear cellulose agar) without their consortial partners, and that in many species both morphological and physiological characteristics undergo gradual changes as these organisms are transferred *in vitro*. For example, SEM of partly digested cellulose in enrichment cultures from both the cattle and the buffalo, and phase contrast microscopy of fresh isolates showed a predominance of cellulolytic Gram-negative rod-shaped cells of *F. succinogenes* as a mixture of cells with regular shape and various sizes. As pure cultures of these organisms are transferred *in vitro*, regular examination by phase contrast microscopy reveals that they become gradually smaller and more polymorphic until they reach the smaler and irregular shape in adapted laboratory cultures.

The method for the isolation of cellulolytic rumen bacteria used in this study leads to a high recovery of these organisms, and these data agree well with those obtained by direct morphological examination. Data presented suggested that since direct plating on cellulose agar roll tubes does not enable to detect pure colonies of *F. succinogenes*, the presence of this species is underestimated, and it is difficult to isolate it without contamination by *Treponema* and/or *Butyrivibrio* species. In contrast, the selective isolation method we developed allows for the recovery of pure cultures of *F. succinogenes*, and the cellulolytic activity of all species, or contaminations of species, can be accurately assessed in our liquid cellulose detection medium. The combined use of morphological and physiological method with novel isolation techniques is a new approach for gaining a better understanding of the microbial ecology of cellulose digestion in the rumen.

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反すう家畜第一胃からの繊維強化法による繊維分解菌の選択分離と性状

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摘 要

ワットマンセルロース濾紙を唯一の選択基質とする強化培地を用いる方法は反すう家畜のルーメン（第一胃）から繊維分解菌を分離するのに極めて効果的であった。ルーメン試料は非選択グルコース－セロビオース寒天ロールチューブに接種する前に，強化液体培地中で36～96時間培養された。カナダでの実験では，釣菌した120株中，45株が繊維分解菌であった。マレイシアの水牛では，90株中36株が繊維分解菌であった。同様に日本での実験では，420株中，260株が繊維分解菌であった。アルゼンチンでの実験では，16株中10株が繊維分解菌であった。これらの繊維分解菌の大半はファイブロバクター サクシノジェネスであった。ルミノコッカス フラヴェファシエンスは小数出現し，ルミノコッカス アルブスは分離されなかった。トレポネマとの混菌は見られなかったが，ビューチリビブリオとの混菌がかなり観察された。本報に掲載した方法は，これまでの従来法のセルロース寒天培地法や可溶性炭水可物寒天培地法と比較して，効率的に短時間で出来，従来法のセルロース寒天法では，クリアー ゾーンを形成することが出来ない為，分離が出来なかったファイブロバクター サクシノジェネスを繊維分解能を保持したまま分離出来ると言う利点があった。

キーワード：ファイブロバクター サクシノジェネス，ルミノコッカス フラヴェファシエンス，ビューチリビブリオ フィブリソルベンス，トレポネマ，牛，水牛

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