Cellulosome-Like Structures in Ruminal Cellulolytic Bacterium *Ruminococcus albus* F-40 as Revealed by Electron Microscopy

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ABSTRACT : This study provides electron microscopic evidence for the presence of cellulosome-like structures on the cell surface of *Ruminococcus albus* F-40. Electron microscopy showed that clusters of tightly packed spherical particles were located on the cell surface of *R. albus*. The protuberant structures present mainly on the bacterial surface and also bound to the cellulose substrate appeared to be the site of cellulosome-like structures. From the evidence presented, we suggest that the structures described here might be a characteristic feature of some ruminal cellulolytic bacteria. ***(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 10 : 1429-1433)***

Key Words : Cellulosome-Like Structures, *Ruminococcus Albus* F-40, Ultrastructure

INTRODUCTION

The degradation of cellulosic substrates, the dry matter digestibility and the adhesion rate of bacteria to the substrates increased potentially with the decrease of relative crystallinity of cellulose, indicating the preferential breakdown of amorphous cellulose by this bacterium as in table 1. Although the characterization of enzyme components in ruminal cellulolytic bacteria (RCB) is being intensively investigated presently, the cellulolytic enzymes of RCB are not as well understood as fungal enzyme systems. Therefore, the concept of cellulosome described originally in the non-ruminal anaerobic bacterium *Clostridium thermocellum* has been extended to characterize the structural organization and catalytic mechanism of RCB. In *C. thermocellum*, a battery of enzymes organized into an extracellular complex called the cellulosome (Lamed and Bayer, 1988) helps in bacterial adhesion to the cellulosic substrates and efficient cellulolysis. The cellulosomes which are present extracellularly and in association with bacterial cells are regarded to be a characteristic feature of the anaerobic cellulolytic bacteria (Bayer and Lamed, 1986; Felix and Ljundahl, 1993).

However, there is no general agreement on the presence of a cellulosome-like structures in the ruminal bacteria. Based on biochemical and electron microscopic investigations, it has been reported that RCB have cellulosomes (Stack and Hungate, 1984; Mayer et al., 1987; Huang and Forsberg, 1990). But these structures have not been well characterized, and evidence for a cellulosome-like structure from genetic analysis is also lacking. White et al. (1996) reported that all the currently characterized cellulases from RCB lack identifiable docking sequences, and cellulose binding domains (CBD) which are present in the cellulosome of *C. thermocellum*. From the sequence analyses of cellulase and xylanase genes from RCB, these workers concluded that the organization of the fibrolytic enzyme systems of the RCB differed greatly from the cellulosome model proposed for *Clostridium*. In contrast, Karita et al. (1996) cloned a gene encoding family 9 cellulase from *Ruminococcus albus* F-40 and found from sequence analyses that the enzyme consisted of 3 domains, family 9 cellulase, family IIIb CBD, and dockerin-like reiterated sequence. Kirby et al. (1997) also reported dockerin-like sequences in cellulases from *R. flavefaciens*. However, it remains to be determined whether or not *R. albus* produces a clostridial type of cellulosome.

Electron microscopic evidence has presented (Bayer and Lamed, 1986; Mayer et al., 1987) the existence of protuberant structures on the surface of *Clostridium* cells which were grown on cellulose substrate. Use of cyto- and immunochemical techniques in conjunction with electron microscopy suggested that the protuberant structures represent an aggregation of cellulosomes, that may have a role in cellulose degradation.

In this context, it is relevant to identify ultrastructually the cellulosome-like complexes in the ruminal bacteria. This study was undertaken to examine the occurrence of the cellulosome-like structures in RCB using cationized ferritin in conjunction with transmission electron microscope (TEM) and scanning electron microscope (SEM). We observed the surface of *R. albus* cells, one of the predominant cellulolytic bacterium in the rumen, which have protuberant structures similar to those described for *Clostridium*.

MATERIALS AND METHODS

Chemicals.

All chemicals used for TEM and SEM investigations were purchased from Sigma Chemical Co. (St. Louise, Mo.).

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Table 1. Changes of relative crystallinity index, dry matter digestibility and adhesion rate after incubation with *R. albus* F-40[a]

Sustrate	Relative Crystallinity (%)		Digestibility (%)	Adhesion rate (%)
	Before incubation	After incubation		
Rice straw	47.6	47.4	33.7	100
NaOH-treated rice straw	42.9	38.1	46.8	196
Birch wood	48.1	50.9	10.4	50
Birch holocellulose	59.1	55.0	62.4	300

[a] Referenced from Kim et al. (1997a) and Kim et al. (1997b)

Bacterial strain, culture medium and conditions.

The bacterial strain used in this study was *R. albus* F-40 was cultivated under anaerobic conditions at 37°C in cellulose-containing medium as described previously (Ohmiya et al., 1985). Pure cellulose (3% suspension of KC flock, W-300, from Sanyo Kokusaku Pulp, Tokyo, Japan) was ball-milled for 3 days, and then used as the main carbon source in the medium for *R. albus*. As in anaerobic procedures, the culture medium was treated with a stream of nitrogen gas, and the bottles were sealed with butyl rubber septum-type stoppers.

Electron microscopy.

Small pieces of rice straws pre-treated with sodium hydroxide and delignified sawdust of birch wood (1.0%, w/v) were incubated with the *R. albus* F-40 cells anaerobically. Samples were harvested after two-days' incubation and treated with cationized ferritin (CF) ($mg \cdot ml^{-1}$ in 1% NaCl, v/v) for 1 h at room temperature. Subsequently samples were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in cacodylate buffer (pH 7.4) overnight at 4°C. The fixed materials were washed with same buffer, dehydrated through a graded ethanol series and embedded in London resin white. Ultrathin sections (80-100 nm thick) collected on nickel grids (300 mesh) were stained with 4% uranyl acetate or with ammonium molybdate and bacitracin (Knutton 1995). As a control some specimens were fixed without the treatment of cationized ferritin. Sections were examined with a Phillips 300 and JEOL 1010 TEM operating in the range of 60-80 KeV. Measurements of particle sizes were made from prints at calibrated magnifications ranging from ×100,000 to 200,000. For SEM, a solution of CF was applied to samples, and then fixed with 4% glutaraldehyde. After dehydration, samples were substituted with t-butyl alcohol and then freeze dried. Samples were coated with gold and observed under a Hitach SEM.

RESULTS AND DISCUSSION

Transmission electron microscopy observations of ultrathin sections of *R. albus* F-40 stained with cationized ferritin showed nodular structures on the cell surface (fig. 1), and SEM observations showed these structures as surface protuberances in large numbers (fig. 2). These structures were more prevalent when cells were grown in media containing cellulose rather than cellobiose. Various surface

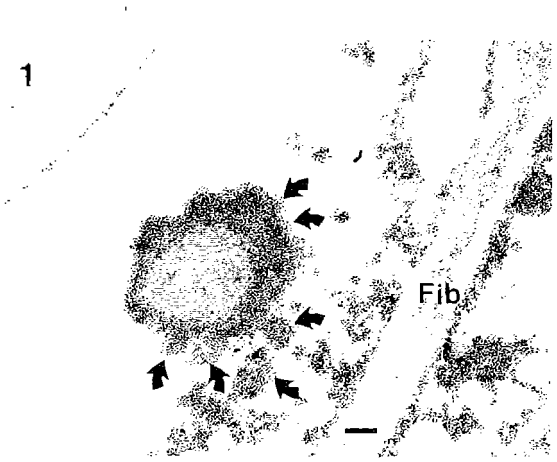

Figure 1. Transmission electron micrograph (TEM) of an ultra thin section of *Ruminococcus albus* F-40. Cells were treated with cationized ferritin (CF) prior to processing for electron microscopy. Note the protuberant structures which decorate the bacterial cells. Some clusters were detached from the bacterial cell and bound to the fibers of rice straw (Fib). Bar = 0.1 μm.

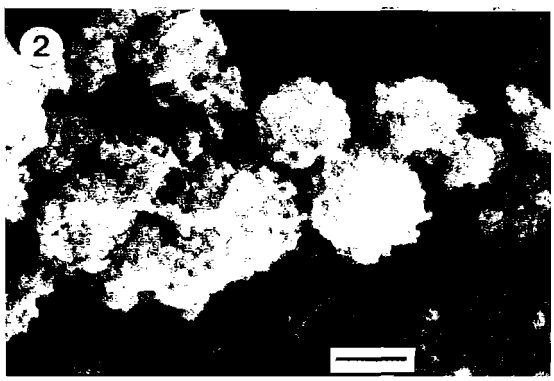

Figure 2. SEM micrograph of the protuberant structures densely coating the bacterial cells of *R. albus* F-40. Cells were stained with CF prior to processing for SEM observation. Bar = 1 μm.

structures associated with *R. albus* cells have previously been observed with TEM (Lamed and Bayer, 1988; Stack and Hungate, 1984; Wood et al., 1982) and from cultures of *R. albus, F. succinogenes,* and *R. flavefaciens* with SEM (Miron et al., 1989), although these cellulosome-like structures were not characterized in any detail.

The dense coat of protuberant structures in *R. albus* F-40 resembled cellulosomes observed in *C. thermocellum* by Bayer and Lamed (1986). Therefore, we examined the ultrastructural feature of the protuberant structures by TEM in greater details, with particular emphasis on the interface between the cell surface and cellulosic substrates.

The use of cationized ferritin revealed the presence of clusters of tightly packed spherical particles on the cell surface of *R. albus* F-40 (fig. 3). Transmission electron microscopy clearly showed cellulosome-like structures within a nodulous protuberant structure on the cell surface of *R. albus* F-40. According to Lamed and Bayer (1988) several hundred cellulosomes are present in each of these protuberances. Transmission electron microscopy observations suggest that each of these clusters has a very large enzyme complex, with a high molecular weight. Mayer et al. (1987) estimated that polycellulosomes (clusters of cellulosomes) in *C. thermocellum* had a particle mass of 50-80$\times 10^6$ daltons, while that of the cellulosomes was estimated to be 2-2.5$\times 10^6$ dalton.

The polycellulosome-like structures superficially resemble grapes in a bunch (fig. 4). Closer examinations of these structures revealed that the clusters appeared to be composed of smaller spherical units (figs. 2, 3, and 4). Although the size of clusters appeared to be variable, the small spherical particles in the clusters were nearly constant with a diameter of 15-20 nm. Large-size clusters in *R. albus*

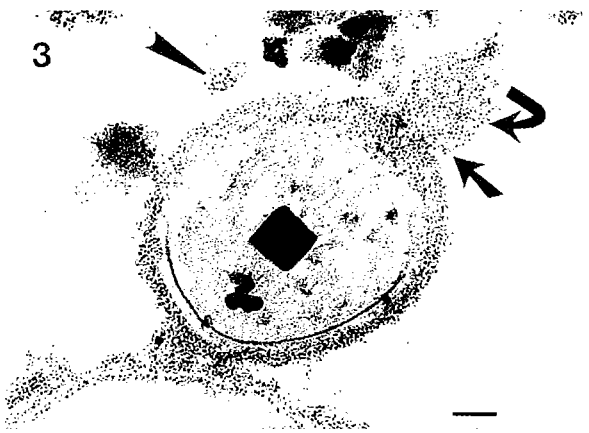

Figure 3. Clusters of cellulosome-like structures located at cell surface of *R. albus* F-40. Note the cluster consists of large number of spherical particles (double arrows). A cluster consisted of relatively small number of spherical particles (arrowhead) detached from the bacterial cell. Bar = 0.05 μm.

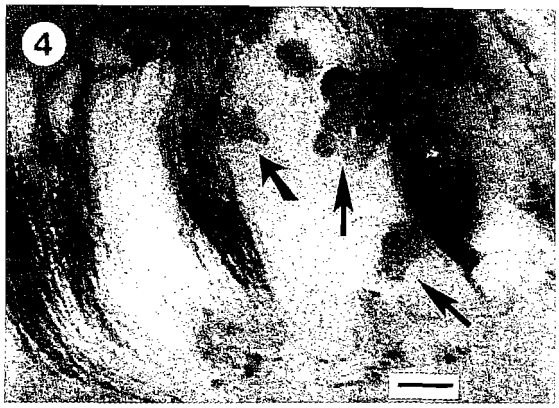

Figure 4. A cluster of small spherical particles (arrowhead) attached to the cellulose microfibrils of birch wood. Cellulose microfibrils were stained with ammonium molybdate. The cluster superficially resembles bunch of grapes. Bar=0. 05 μm.

F-40 were 80-120 nm in diameter (figs. 3 and 4). It is interesting to note that clusters composed of relatively small number of spherical particle with diameter ranging from 25 nm to 40 nm were found detached from the bacterial cell surface (fig. 3 and 4), suggesting desegregation of large high molecular complexes into smaller ones. Glucanases in general are assumed to be on the cell surface of ruminal bacteria (Flint and Forsberg, 1995). Mayer et al. (1987) noted that the shape of cellulosomes changed from the tightly packed complexes to loosely packed complexes and ultimately free polypeptides, depending upon the stage of cultivation. Coughlan et al. (1985) detected three major globular complexes with a diameter of 21 nm and larger forms of 35-45 nm and 61 nm in *C. thermocellum* by electron microscopy. These observations suggest that variations in the size of cellulase packages are likely to occur in relation to bacteria, mode and stages of cultivation and possibly the cellulosic substrate.

The clusters of the spherical particles were also seen bound to cellulose microfibrils (figs. 4 and 5). TEM showed that the cellulose fibers were covered with spherical particles (fig. 5), suggesting that bacterial adherence to and breakdown of the cellulosic substrate is mediated by cellulosome-like structures in *R. albus* F-40. This is consistent with the observations of Beguin and Lemaire (1996) who reported that some proteins in the cellulosome contain binding domains, which facilitate a physical contact between bacterial cells and the cellulosic substrates.

Mayer et al. (1986) found that when bacterial cells came in contact with cellulose, some protuberances protracted, yielding a fibrous network. Such fibrous structures were not observed in the present study of *R. albus* F-40. Instead, we observed the extracellular structures of bacterial cells anchored to the cellulosic substrates (fig. 6). However, we have yet to confirm that the structures found in *R. albus* F-

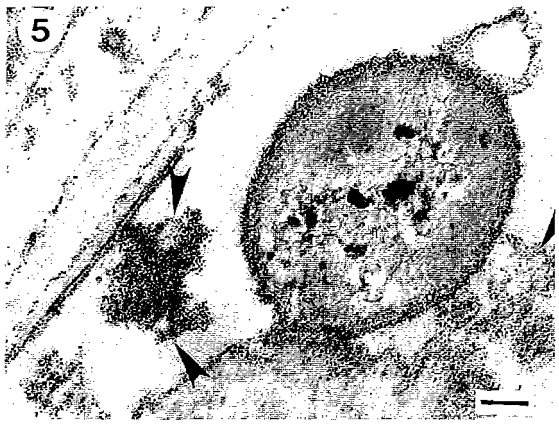

Figure 5. Clusters of spherical particles attached to the fibres of rice straw. Some areas of the cellulose fibers (F) are covered by free cellulosome-like structures of different sizes. Bar = 0.1 μm.

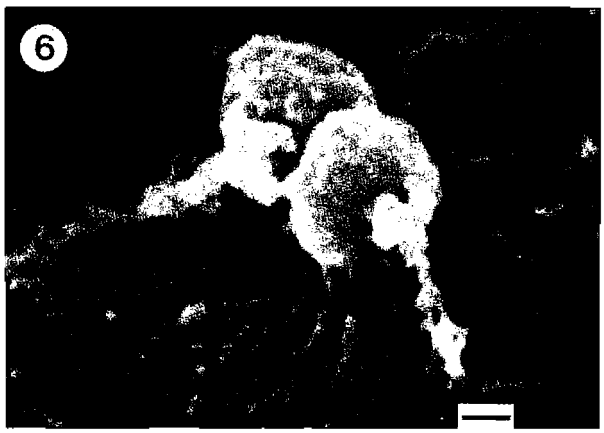

Figure 6. The extracellular surface structures of *R. albus* anchored to the cellulosic substrate. Cells were treated with cationized ferritin (CF). Note the protuberant structures. Bar = 0.2 μm.

40 and those observed by Mayer et al. (1986) share characteristics which are common to both. Further studies should be aimed to be determined whether or not the spherical particles have cellulase activities.

When compared to the cellulosome-like structures, the vesicles either attached to or released from bacteria were of variable sizes (fig. 7). Furthermore, a cluster of tightly packed spherical particles observed in the cellulosome-like structures was not found in the vesicles.

In conclusion, the results of TEM investigations showed that cellulosome-like structures in *R. albus* F-40 are cell-associated and cellulose-binding. The present study strongly suggested that cellulosome-like structures might be a characteristic feature of *R. albus* F-40. Further studies are needed to clarify whether the cellulosome-like structures have cellulolytic activities and the cellulosome concept can be applied to all RCB. In addition, further insight into the characteristics of cellulosome-like structure is also needed to more precisely understand the degradation mechanisms of cellulose by RCB. Some progress to characterize the enzymes of cellulosome-like structures in *R. albus* F-40 has already been made by Karita et al. (1996), who sequenced the cellulase gene from *R. albus* F-40 and showed that *R. albus* produced a clostridial type of cellulosome.

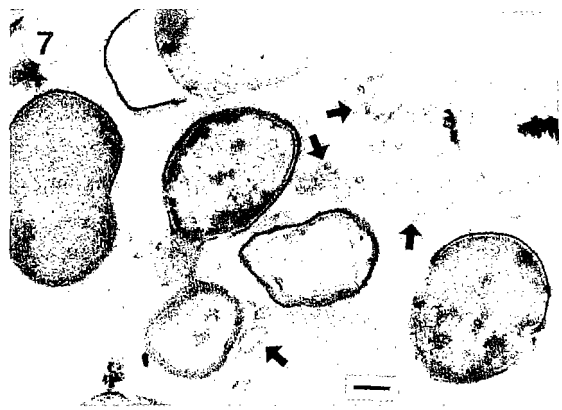

Figure 7. Extracellular vesicles around the bacterial cells. Note the various size of vesicles. Bar = 0.1 μm.

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