Preservation of Ruminal Bacterium Capsules by Using Lysine in the Electron Microscopy Fixative

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Received 2 April 1990/Accepted 25 June 1990

Ruminal bacteria from axenic cultures of Ruminococcus flavefaciens FD1, Butyrivibrio fibrisolvens 49, and bacterial types from the ruminal ecosystem that were fixed with 50 mM lysine (L-lysine hydrochloride) added to glutaraldehyde had better-preserved capsules and extracellular material than bacteria fixed without lysine.

Transmission electron microscopy (TEM) has been used to study the external polysaccharide material (capsules and slime) produced by bacteria (9), including capsular features of bacteria associated with plant cell walls during degradation (3). Cheng and Costerton (5) stated that all bacteria in a study of organisms from ruminal fluid of cows fed hay possessed an external "coat" layer, and these researchers reported 10 different types of coat layers.

The hydrated nature of the bacterial capsule makes its preservation during electron microscopy difficult and subject to shrinkage and artifacts (7, 9). Several methods, including ferritin-labeled antibodies and ruthenium red, have been used to preserve and aid in the investigation of capsular structure by TEM (9). Recently, Jacques and Graham (7), using a technique reported and evaluated by Boyles et al. (4), incorporated lysine into glutaraldehyde fixative and observed improved preservation of bacterial capsules. These researchers (4) suggested that since lysine and glutaraldehyde could form large polymers having net positive charges at physiological pHs, this diamine may cross-link with bacterial capsules and thus stabilize the capsule during subsequent steps for electron microscopy preparation. We used this method to study capsules of axenic cultures of ruminal bacteria and mixed bacteria from the ruminal ecosystem associated with plant fiber.

Preparation of ruminal bacteria for TEM. Pure cultures of Ruminococcus flavefaciens FD1 and Butyrivibrio fibrisolvens 49 were obtained from S. A. Martin, Department of Animal and Dairy Science, University of Georgia, Athens. These organisms were maintained on basal medium (2) containing 0.2% (wt/vol) cellulose or leaf and stem segments of Bermuda grass or alfalfa to maintain the fiber-degrading enzymes. For TEM studies, R. flavefaciens cells were inoculated into medium with alfalfa leaf and stem sections and incubated for 48 h. Cells of B. fibrisolvens grown for 48 h in cellulose broth were mixed with 2% molten agar, which was allowed to harden on a glass surface. Blocks (2 mm3) were excised from the hardened agar. For mixed bacteria from the ruminal ecosystem, basal medium containing 3-mm leaf sections of Bermuda grass was inoculated with 0.2 ml of strained (through cheesecloth) ruminal fluid from a steer eating Bermuda grass hay.

Based on a protocol reported by Jacques and Graham (7), plant fragments or agar squares with bacteria were prepared for TEM as follows: (i) fixed in freshly prepared 2.5% (vol/vol) glutaraldehyde containing 0.075% (wt/vol) ruthenium red and 50 mM lysine (L-lysine hydrochloride) in 0.1 M sodium cacodylate buffer (pH 7.4) for 20 min at room temperature; (ii) fixed for 100 additional min at room temperature in the above solution except that lysine was omitted; (iii) washed three times for 5 min each in 0.1 M cacodylate buffer (pH 7.4); (iv) postfixed in 1.5% (wt/vol) OsO4 in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 8°C; (v) washed three times for 5 min each with 0.1 M cacodylate buffer and stored in buffer overnight at 8°C. Control plant pieces and agar squares were fixed without lysine by omitting the first step above. Plant fragments and agar squares fixed with or without lysine were dehydrated through a graded ethanol series (50, 70, 90, 100% [vol/vol] three times), infiltrated in an ethanol-Spurr (10 mixture (2:1; 1:1; 1:2) for 1 h each step, infiltrated in 100% Spurr for 1 h, embedded in fresh 100% Spurrs, and cured at 70°C for 60 h. Ultrathin sections were stained with uranyl acetate and lead citrate as described previously (3).

Bacterial capsules and extracellular structures were evaluated with a JEOL JEM-100 CXII transmission electron microscope. From electron micrographs, prints (total magnification of 828,000) were made of each of the organisms and treatments. The average thickness from the cell membrane to the edge of the capsule or extracellular material was determined at two to four sites by using a digitizer. Bacteria in oblique sections were not measured, since the size of the extracellular material would be distorted. For the fiber-degrading bacteria, only sites not associated with plant walls were measured since the capsule was often elongated at the point of attachment. In the mixed bacterial population, bacteria evaluated included (i) encapsulated Ruminococci, (ii) regularly shaped bacteria (2), and (iii) irregularly shaped bacteria (2). Thicknesses of extracellular structures were compared for bacterial types prepared by the two fixation procedures by using the Student t test.

Incorporation of lysine into the glutaraldehyde resulted in a statistically (P < 0.01) thicker extracellular structure for every bacterial type evaluated (Table 1). R. flavefaciens had the largest capsule regardless of treatment, and lysine-glutaraldehyde resulted in a capsule at least twice as thick as that fixed in glutaraldehyde alone (Table 1; Fig. 1). Thin filaments which appeared to be part of the extracellular material stretched between bacteria and other objects (e.g., plant walls) (Fig. 1, arrowheads) in both fixation procedures.

In B. fibrisolvens, distinct capsules or other extracellular materials were rarely observed in specimens fixed without lysine (Table 1; Fig. 2), but incorporation of lysine resulted

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in observable extracellular structures. Often, the extracellular substance appeared as loosely organized material or as "knobby structures" similar to those of B. fibrisolvens D1 reported by Cheng and Costerton (6).

In mixed populations, encapsulated ruminococci similar to \textit{R. flavaeaciens} possessed an enhanced capsule when fixed with lysine (Table 1; Fig. 3). The capsule facilitated attachment of bacteria to plant cell walls during degradation of this substrate. The regularly shaped bacteria, while not distinctive of a particular organism and possibly representative of several, had a loosely organized or knobby surface that was preserved by lysine-glutaraldehyde fixation (Fig. 3). Irregularly shaped bacteria (Fig. 3) did not possess a distinctive capsule like the ruminococci, but the loosely organized, extracellular material was better preserved with lysine fixation (Table 1). The pleomorphic nature of similar, gram-negative organisms attached to fiber indicated that these bacteria most likely represented \textit{Fibrobacter succinogenes} (8). At the point of attachment (Fig. 3, inset), the extracellular material was not distinguishable.

Earlier research (1) by TEM had shown that extracellular structures of ruminal bacteria were more electron dense with ruthenium red, but sizes and morphologies were not substantially different from those of structures prepared without ruthenium red. Incorporation of lysine into the glutaraldehyde fixative for electron microscopy preserved stability of the capsule and extracellular material of axenic cultures of ruminal bacteria and of bacteria in mixed populations from the ruminal ecosystem. Different forms of the material occurred with different organisms, and omission of the lysine resulted in lack of observable extracellular material in some

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**TABLE 1. Width of extracellular material fixed with or without lysine**

<table>
<thead>
<tr>
<th>Source of inoculum</th>
<th>Bacterium or type</th>
<th>Thickness (nm) of extracellular material$^{a}$</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Lysine</td>
</tr>
<tr>
<td>Axenic culture</td>
<td>\textit{Ruminococcus flavaeaciens} FD1</td>
<td>178 ± 21$^b$ (n = 50)</td>
</tr>
<tr>
<td></td>
<td>\textit{Butyribrio fibrisolvens} 49</td>
<td>55 ± 0$^b$ (n = 33)</td>
</tr>
<tr>
<td>Ruminal ecosystem</td>
<td>Encapsulated ruminococci</td>
<td>134 ± 15$^b$ (n = 51)</td>
</tr>
<tr>
<td></td>
<td>Regularly shaped bacteria</td>
<td>23 ± 22$^b$ (n = 45)</td>
</tr>
<tr>
<td></td>
<td>Irregularly shaped bacteria</td>
<td>64 ± 30$^b$ (n = 53)</td>
</tr>
</tbody>
</table>

$^a$ Distance measured from membrane to edge of material for two to four sites per bacterium. Values are average and standard deviation and $n$ = number of bacteria measured.

$^b, c$ Values in same row with different superscripts differ ($P < 0.01$).

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**FIG. 1.** \textit{R. flavaeaciens} FD1 fixed without (a) or with (b) lysine. Lysine resulted in a larger capsule. Thin filaments connect the capsule to other materials (arrowheads). Bar, 1 \(\mu\)m.

**FIG. 2.** \textit{B. fibrisolvens} 49 fixed without (a) or with (b) lysine. Extracellular material was preserved in fixative with lysine, appearing as loosely organized material or as knobs (arrowhead) on the bacterial surface. Bar, 1 \(\mu\)m.
organisms and a reduced capsular structure in others. The addition of lysine to the fixation procedure rendered capsules thicker and more dense (compare ec in Fig. 3a and b). This simple procedure should provide for better evaluation of ruminal bacteria in studies, such as those with compounds that stimulate capsules (11), in which emphasis is on the ultrastructure of extracellular material.

We thank S. A. Martin, University of Georgia, for providing axenic cultures from his maintained collection.

LITERATURE CITED