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Effect of 3-Phenylpropanoic Acid on Capsule and Cellulases of *Ruminococcus albus* 8

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The morphology and cellulases of *Ruminococcus albus* 8 were markedly affected by the inclusion of 3-phenylpropanoic acid (PPA) in a defined growth medium. PPA-grown bacteria produced substantial quantities of cell-bound cellulase, as well as a very high-molecular-weight extracellular enzyme and lesser amounts of two low-molecular-weight enzymes. PPA-deprived bacteria produced greater total amounts of cellulase, but all of it exists in soluble, low-molecular-weight forms. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the availability of PPA did not affect the kinds of proteins produced, but the distribution of two major proteins between cells and supernatant was PPA dependent. These two proteins (85 and 102 kilodaltons) were primarily associated with the cells of PPA-grown bacteria but were found chiefly in the supernatants of PPA-deprived cultures. Examination of thin sections of PPA-grown *R. albus* 8 by transmission electron microscopy showed a lobed ruthenium red-staining capsule surrounding the cell wall, as well as small vesicular structures (diameter, 0.05 to 0.06 μm) which appeared to aggregate into larger spherical units (diameter, 0.2 to 0.3 μm). In contrast, thin sections of PPA-deprived cells were devoid of vesicles and showed little or no capsule surrounding the cells.

We have previously reported that 3-phenylpropanoic acid (PPA) dramatically affects the rate of growth and cellulose digestion in cultures of *Ruminococcus albus* 8 (5). Wood et al. (13) have reported that the cellulase complex of *R. albus* SY3 is markedly influenced by culture conditions. When cultured in medium containing rumen fluid, it produced chiefly cell-bound cellulase and an extracellular enzyme of very high molecular weight. Bacteria cultured in semidefined medium produced only soluble, low-molecular-weight enzyme. These authors also reported that compared with the original isolate, the polysaccharide capsule surrounding strain SY3 became much thinner during repeated cultivation.

The results presented in the present study with strain 8 confirm and extend the findings of Wood et al. (13) and attribute the differences in the cellulase complex and capsule to the content of PPA in the growth medium. Some differences between strains SY3 and 8 were observed, however, particularly in the difficulty encountered in extracting cell-bound cellulase from strain 8.

MATERIALS AND METHODS

Culture procedures. *R. albus* 8, employed for all studies, was isolated and maintained on a defined medium (12) containing 0.15% pebble-milled Whatman no. 1 filter paper (PMC) and 25 μM PPA.

One-liter cultures for enzyme analysis were prepared with either 0.15% PMC or 0.15% cellobiose as the carbon source in the presence or absence of 25 μM PPA. These were inoculated with 0.1 ml of a 24-h stock culture of *R. albus* 8 on the defined medium and incubated at 37°C. The cultures containing 0.15% cellobiose without PPA, 0.15% cellobiose with 25 μM PPA, and 0.15% PMC with 25 μM PPA completed growth and were harvested after 4 to 5 days, whereas the culture containing 0.15% PMC without PPA required 4 weeks of incubation.

These media were also used to grow 6-ml cultures for transmission electron microscopy. Each was inoculated with 0.1 ml of a culture of *R. albus* 8 depleted of PPA by inoculating 0.06 ml of stock culture into 6 ml of 0.15% PMC medium lacking PPA. The cellobiose cultures were harvested by centrifugation in mid-log phase (optical density at 620 nm of ~0.20 to 0.25), whereas the cellulose cultures were harvested after approximately one-half of the cellulose had been utilized.

Preparation and fractionation of cellulase. Cultures kept anaerobic by the addition of 4 ml of 2-mercaptoethanol per liter were centrifuged anaerobically under CO_2 at 4°C in Teflon-lined stainless steel centrifuge bottles (Servall, Inc.) at 15,000 × *g* for 60 min. The enzymes in the supernatant were concentrated anaerobically to 6 ml by ultrafiltration over an Amicon PM-10 membrane in an Amicon model 202-stirred cell at 0°C under N_2 at 20 lb/in^2. One milliliter of this enzyme concentrate was loaded onto a column (1.5 by 49 cm) of Fractogel TSK 65W (Merck & Co., Inc.) and eluted with 0.1 M sodium phosphate (pH 6.8) containing 54 mM 2-mercaptoethanol (Pi-ME buffer). The eluant was monitored continuously at 280 nm, and the 1.3-ml fractions collected were analyzed for protein and cellulase activity.

The cell pellet from each 1-liter culture was dispersed in 12 ml of Pi-ME buffer by gentle homogenization in a glass-Teflon tissue homogenizer. Extraction of the cell-bound cellulase was attempted by vigorous homogenization of 2.5-ml samples of the dispersed cell pellet in the Pi-ME buffer at 25°C in buffer containing 2 M sodium chloride at 25°C, in buffer containing 0.3% Triton X-100 (Rohm and Haas, Inc.) at 0°C, or in buffer containing 0.3% Tween 80 (Sigma Chemical Co.) at 0°C.

The homogenized cell suspension was assayed for cellulase as described below, as were the supernatants of these treated pellets after centrifugation at 8,600 × *g* for 10 min.

Cellulase assays. Anaerobic cellulase assay broth was prepared as follows. A total of 250 ml of a 3% suspension of PMC (pebble milled for 24 h) was combined with an equal volume of distilled water containing the following ingredients (in millimoles per liter) in the final medium: KH_2PO_4, 3.0; K_2HPO_4, 3.0; NaCl, 1.5: $(NH_4)_2SO_4$, 1.5; $MgSO_4$, 1.0; and $CaCl_2$, 0.5. After the addition of 0.5 ml of 0.1% (wt/vol)

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resazurin, this mixture was boiled in a 1-liter round-bottom flask to drive off O_2 and then stoppered and cooled in a water bath under a stream of CO_2. Solid $NaHCO_3$ (2.5 g) was added slowly during constant stirring with a small Teflon-coated magnetic stir bar. The assay broth was finally reduced by the addition of 2 ml of 2-mercaptoethanol. Cellulase activity was determined anaerobically under CO_2 at 37°C by the addition of 25- to 100-μl samples (or suitable dilutions) of enzyme to 1.5 ml of the assay broth in disposable glass tubes (13 by 100 mm) capped with no. 00 butyl rubber stoppers. Assay tubes were incubated for 20 to 22 h and then centrifuged at 900 × *g* for 10 min. Solubilized carbohydrate in 0.3-ml samples of the supernatant was measured by the anthrone method (2), with cellobiose as a standard. One unit of enzyme activity was defined as that amount of activity liberating 1 μmol of carbohydrate (as cellobiose) per h at 37°C.

Assays on cell pellets and cell pellet extracts were performed similarly, except that each tube contained in addition 50 μl of a solution of 1-mg/ml chloramphenicol in ethanol to inhibit growth.

Protein assays. Soluble protein was estimated by the Coomassie blue dye-binding technique of Bradford (1), with bovine serum albumin (Sigma) as a standard. For quantitation of protein in cell pellets the following modification was used. The absorbance at 595 nm was measured 30 min after the addition of a 0.1-ml sample (containing dispersed cells) to 2.0 ml of dye reagent. Subtracted from this was the reading at 595 nm obtained from mixing an identical 0.1-ml sample with 2.0 ml of water. This difference was used to estimate protein values from the standard curve; these were found to correlate well with values obtained for total soluble protein measured after extensive sonication of cells.

Transmission electron microscopy. Fixation and staining of cells for transmission electron microscopy were by the basic procedure of Luft (8), as described by Patterson et al. (10). The cells and undigested cellulose (where applicable) from two 6-ml cultures of each condition were collected by centrifugation at 900 × *g* for 10 min and combined. The pellet was suspended in 2 ml of 0.15% ruthenium red for 30 min at 25°C and then centrifuged as described above for 15 min. The supernatant was removed, and the pellet was suspended for 1 h in 2 ml of 1.2% glutaraldehyde in 67 mM cacodylate buffer (pH 7.3) containing 0.05% ruthenium red. The sample was centrifuged again for 15 min, and the pellet was washed three times in 2 ml of 67 mM cacodylate buffer containing 0.05% ruthenium red. The cells were suspended and postfixed for 7 h in 2 ml of 1.33% OsO_4 in cacodylate-ruthenium red buffer, and this was followed by three more washings in cacodylate-ruthenium red buffer. Samples were dehydrated by immersion for 30 min in each of 30, 50, 70, 90, and 100% ethanol; washed twice in 100% propylene oxide; and embedded in epon-araldite epoxy resin by the method of Mollenhauer (9). Ultrathin sections were made with a Sorvall MT-1 ultramicrotome and either examined directly or poststained in uranyl acetate (30 min) and lead citrate (20 min) before viewing on a Zeiss EM-109 electron microscope.

Electrophoresis. Cell pellets in Pi-ME buffer and extracellular enzyme concentrates were made 1% in sodium dodecyl sulfate (SDS) and denatured by immersion in a water bath at 100°C for 10 min. After cooling, the samples were dialyzed against 0.1% SDS and prepared for electrophoresis by the method of Laemmli (6). Electrophoresis was performed on either 10 or 12.5% acrylamide slabs, using chemicals, reagents, and standards purchased from Bio-Rad Laboratories.

TABLE 1. Comparison of protein and cellulase content of *R. albus* 8 cultures grown with and without PPA

Culture	Cell protein[a]	Extracellular protein[a]	Cell PMCase[b,c]	Extracellular PMCase[b]
Cellobiose alone (no PPA)	12.4 (39)	18.9 (61)	4 (3)	149 (97)
Cellobiose with 25 μM PPA	16.9 (53)	15.3 (47)	26 (34)	49 (66)
Cellulose alone (no PPA)	10.9 (33)	22.2 (67)	6 (2)	291 (98)
Cellulose with 25 μM PPA	19.1 (64)	10.5 (35)	47 (41)	66 (59)

[a] Measured in milligrams per liter of culture; values in parentheses indicate percentage of total protein.

[b] Activities expressed are based on initial rates of hydrolysis with PMC as a substrate. Soluble carbohydrate detected by anthrone is converted to cellobiose equivalents, and values listed are in units of micromoles of cellobiose hydrolyzed per hour per liter at 37°C; values in parentheses indicate percentage of total PMCase.

[c] Values listed for the PPA-deprived cultures are the values obtained in the presence of Triton X-100.

RESULTS

Liter cultures of strain 8 on 0.15% cellobiose with and without PPA incubated at 37°C without shaking showed considerable differences during growth. The culture with PPA became increasingly turbid (maximum optical density at 620 nm, ~0.7), with most cells dispersed uniformly throughout the culture flask. In contrast, cultures grown without PPA became only slightly turbid, with large cell aggregates accumulated at the bottom of the flask. Even after thoroughly dispersing these cells by vigorous shaking, aggregates tended to reform and accumulate at the bottom of the flask within 30 min. The culture grown with PPA also reached maximum growth in less time (~16 h) than the culture without PPA (~30 h).

Digestion of cellulose was rapid and essentially complete within 3 days in the liter cultures of strain 8 grown on 0.15% PMC with PPA. Without PPA, ca. 4 weeks of incubation were needed for complete digestion of the cellulose. Digestion of PMC in PPA-deprived cultures seemed to proceed in two stages; one-fourth to one-third of the settled volume of cellulose was digested within 3 to 4 days, and the remainder was slowly hydrolyzed over the remaining 3 to 4 weeks.

The yields of cellulase and protein obtained with each of the four culture conditions are presented in Table 1. Although the total yield of protein per liter was more or less independent of nutritional status, much more of this protein was cell associated when cultures were grown with PPA. Concomitantly, these cells contained substantial quantities of cell-bound cellulase. In contrast, cells grown without PPA contained very little cell-bound cellulase, and a higher fraction of the total protein produced under these conditions was extracellular.

Triton X-100 or Tween 80 at a concentration of 0.3% (wt/vol) did not affect the activity of either the cell-associated or extracellular cellulases produced by PPA-grown cells. Similarly, the extracellular cellulases produced by PPA-deprived cells were unaffected by detergent treatment. However, the small amount of cellulase activity associated with PPA-deprived cells was 1.5- to 2-fold greater when assayed in the presence of detergent. This increased cellulase activity probably resulted from better dispersion, as the detergent-treated PPA-deprived cells no longer formed the aggregates mentioned above.

TABLE 2. Amounts of cell-bound cellulase activity extracted by various treatments of *R. albus* 8 cells

Culture	Total pellet PMCase[a]	Amt[a] (%) extractable with:			
		0.3% Triton X-100	0.3% Tween 80	2 M NaCl	Pi-ME buffer (alone)
Cellobiose alone (no PPA)	4	1.8 (46)	1.8 (46)	0.9 (23)	1.2 (29)
Cellobiose with 25 μM PPA	26	2.9 (11)	2.9 (11)	2.1 (8)	2.1 (8)
Cellulose alone (no PPA)	6	2.8 (47)	2.8 (47)	0.6 (10)	0.9 (15)
Cellulose with 25 μM PPA	47	13.2 (28)	13.2 (28)	8.5 (18)	9.9 (21)

[a] Values listed are in units of micromoles of cellobiose equivalents per hour per liter at 37°C.

The amount of cell-associated cellulase extracted after homogenization of cell pellets in variously supplemented buffer is shown for each culture in Table 2. Although 45 to 50% of the small amount of cellulase associated with the walls of PPA-deprived bacteria could be solubilized in the presence of 0.3% Triton X-100 or 0.3% Tween 80, the absolute amount was less than for the PPA-grown cells, in which a maximum of 28% was solubilized.

Gel filtration of the extracellular enzyme concentrates on Fractogel TSK 65W yielded the results shown in Fig. 1. Strain 8 produced a cellulase complex of very high apparent

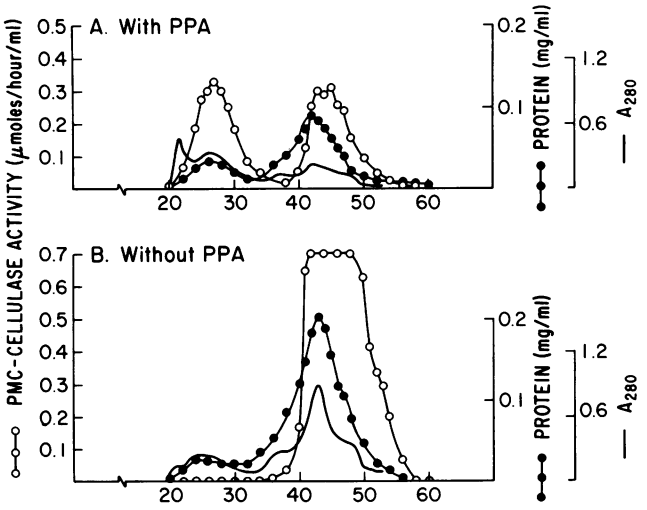

FIG. 1. Results of gel filtration on Fractogel TSK 65W of the extracellular enzymes from *R. albus* 8 grown with and without PPA. Protein and cellulase activity was measured in 100-μl samples of the 1.3-ml fractions as described in the text. (A) Profile from culture grown on 0.15% cellobiose with 25 μM PPA. (Profile from culture grown on 0.15% cellulose and 25 μM PPA was similar.) (B) Profile from culture grown on 0.15% cellobiose without PPA. (Cellulose-grown PPA-derived culture was very similar.) A_{280}, Absorbance at 280 nm.

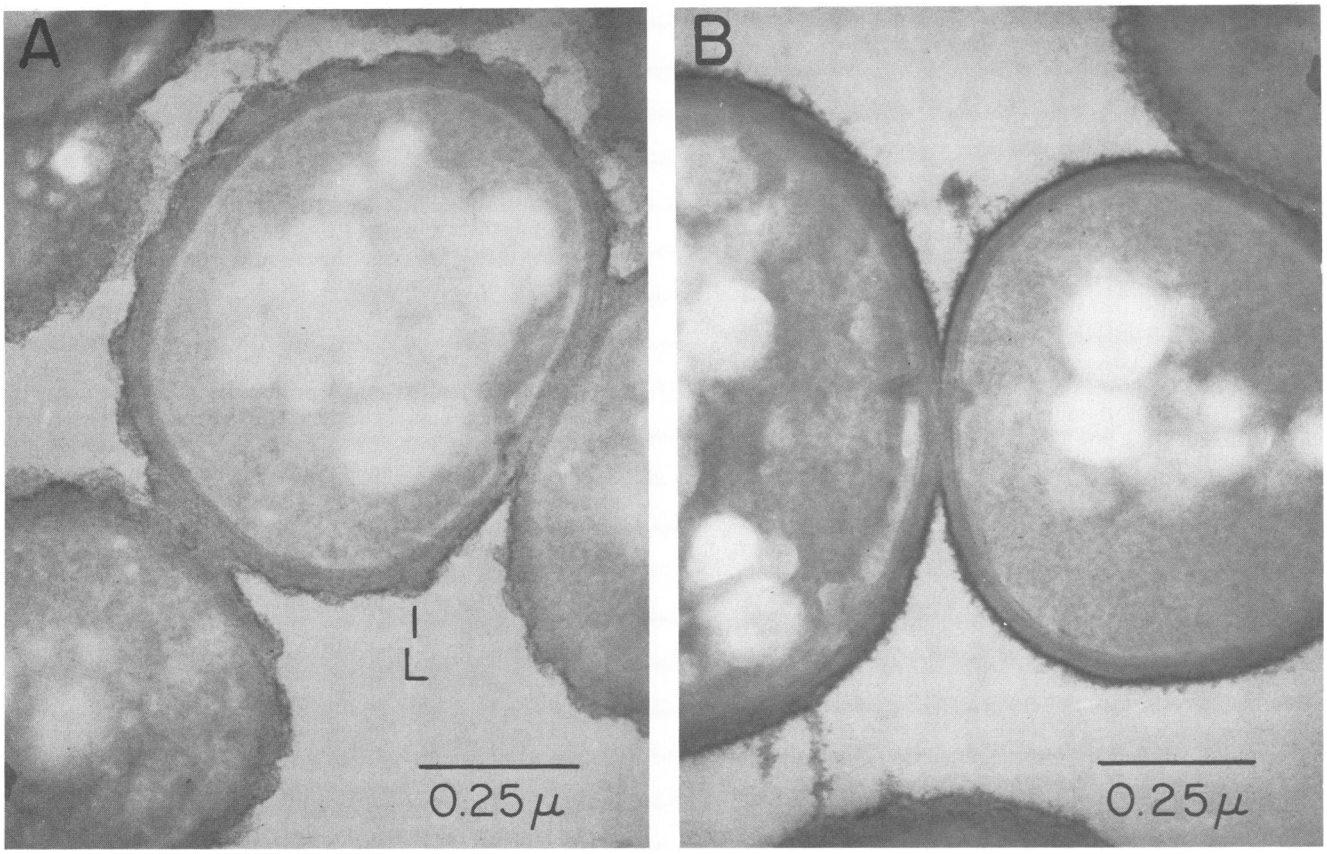

FIG. 2. Transmission electron micrographs showing the surface detail of *R. albus* 8 cells grown on cellobiose and stained with ruthenium red. (A) Cells from cultures supplemented with 25 μM PPA. Note the lobed structures (L) on the surface of the cells. (B) Cells from cultures without PPA. Bar marker represents 0.25 μM.

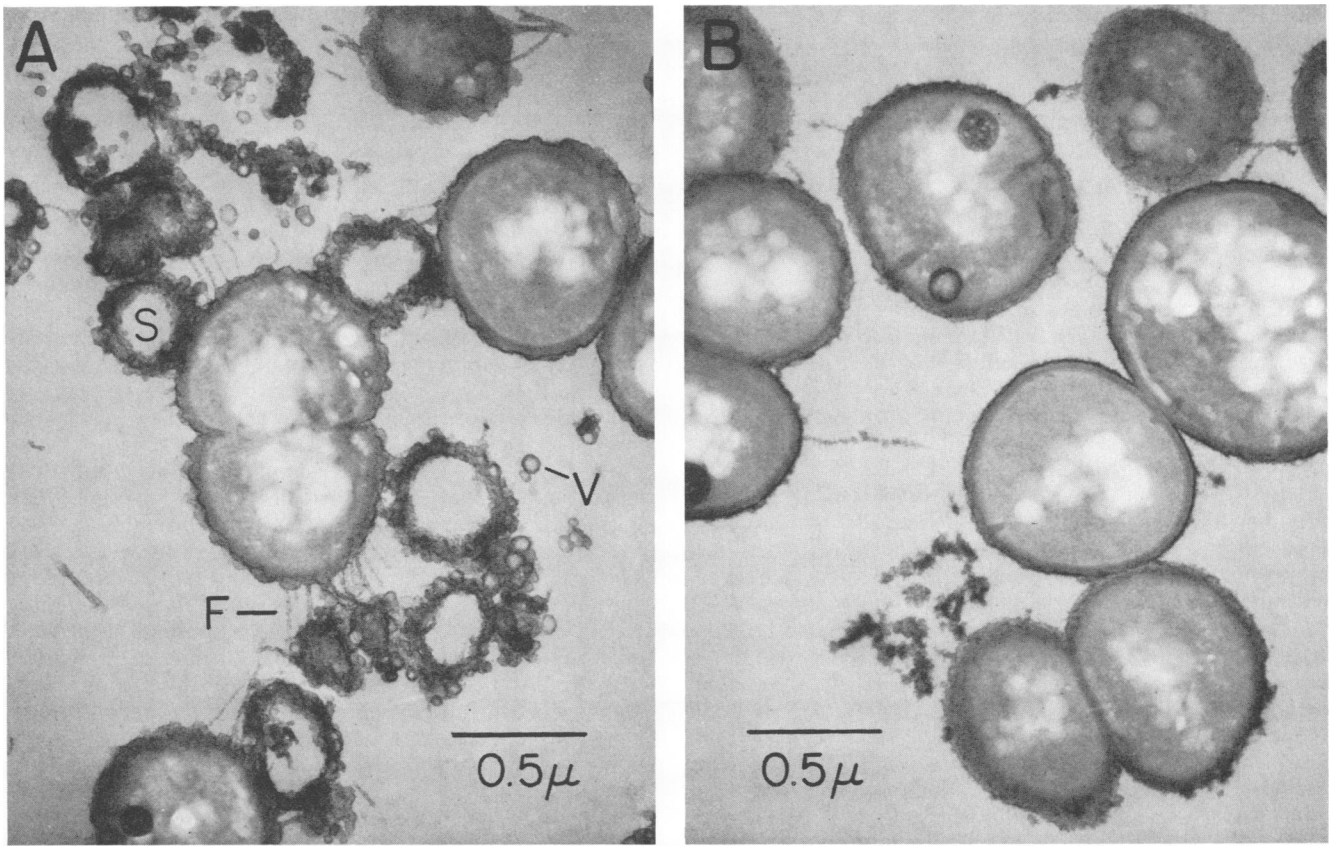

FIG. 3. Transmission electron micrographs of thin sections of cellobiose-grown *R. albus* 8 cells stained as in Fig. 2 and shown at lower magnification. (A) Field of cells from culture supplemented with 25 μM PPA. Note vesicular structures (V) which appear to aggregate into spherical bodies (S). Fibrils (F) are also observed. (B) Cells from culture without PPA. Bar marker indicates 0.5 μm.

molecular weight (as well as lesser quantities of two lower-molecular-weight enzymes) when grown with PPA, regardless of carbon source (Fig. 1A). None of this large-molecular-weight enzyme was found when cultures were deprived of PPA; all of the extracellular enzyme was of lower molecular weight (Fig. 1B).

Thin sections of cells grown under the various conditions described above were examined by transmission electron microscopy. An extensive, lobed capsule surrounded the cell wall of PPA-grown cells (Fig. 2A), in contrast to PPA-deprived cells devoid of capsule (Fig. 2B). This difference was independent of the carbon source for growth (PMC or cellobiose). Figure 3A shows not only the capsule of PPA-grown cells, but also numerous vesicular structures (diameter, 0.05 to 0.06 μm), occasionally single but usually aggregated into spherical structures with diameters ranging from 0.2 to 0.3 μm. None of these structures were observed in thin sections of PPA-deprived cultures (Fig. 3B).

Results of SDS-polyacrylamide gel analysis of fractions from the four cultures are shown in Fig. 4. Although no additional proteins were induced by the presence of PPA, the distribution of two major protein components (85 and 102 kilodaltons [kD]) was PPA dependent. In both PPA-grown cultures, these two components were found primarily in the cell pellets. In both PPA-deprived cultures, these components were found chiefly in the supernatant.

DISCUSSION

In contrast to the uniform turbidity of PPA-grown cultures, PPA-deprived cellobiose cultures of *R. albus* 8 showed large cell aggregates which tended to reform rapidly even after vigorous shaking. Similarly, Wood et al. (13) have reported that *R. albus* SY3 cells did not separate readily when grown on semidefined medium. These observations indicate significant differences in the surface properties of *R. albus* attributable to PPA.

Examination of Table 1 shows little difference in the total amount of protein produced by *R. albus* 8 in response to different culture conditions. However, in PPA-grown cultures the fraction of the protein and cellulase activity in the cell pellet was much greater than in the supernatant. In contrast, PPA-deprived cultures contained nearly all of the cellulase activity and most of the protein in the supernatant.

Although PPA did not affect the kinds of proteins produced, SDS-polyacrylamide gels showed that the distribution of two major proteins of apparently 102 and 85 kD (Fig. 4) was PPA dependent. These two proteins predominated in the cell pellets of PPA-grown cultures, whereas they were chiefly extracellular constituents of PPA-deprived cultures. Although distributed concomitantly with the cellulase activity, these two proteins cannot be the smaller cellulase enzymes (45 and 23 kD; R. Stack, unpublished data) because of the marked difference in molecular size. All of these differences in the distribution of proteins and cellulase activity show that PPA has significant influence on the surface structure of *R. albus* cells.

R. albus cells examined by transmission electron microscopy did show important surface differences, depending on the availability of PPA. PPA-grown bacteria were surrounded by an extensive, lobed capsule absent in PPA-deprived

cells (Fig. 2). The necessity for PPA may be due to its inclusion as a component of the capsule. The results in Table 1 and Fig. 4 suggest that the capsule contains cellulose activity and other proteins of unknown function.

In addition to these surface differences, the availability of PPA had significant effects on the extracellular enzymes produced by *R. albus*. Gel filtration of these enzymes from PPA-grown cultures showed a very high-molecular-weight cellulase in addition to lesser quantities of two low-molecular-weight cellulases. PPA-deprived cultures produced only large quantities of the two low-molecular-weight enzymes.

Since PPA causes no demonstrated difference in the kinds of proteins produced (Fig. 4), the cellulase activity of the large enzyme must be due to inclusion of the low-molecular-weight cellulases (and possibly other proteins) into a complex whose formation depends on the availability of PPA. The vesicular structures (diameter, 0.05 to 0.06 μm) or their larger spherical aggregates (diameter, 0.2 to 0.3 μm) (Fig. 3A) may represent these enzyme complexes. The lobes extending from the surface of PPA-grown cells (Fig. 2A) may represent stages in the process of vesicle formation, since their diameters are similar. The large enzyme may be likened to a raft of capsular material, carrying multiple enzymes.

R. albus and other rumen bacteria have frequently been reported to adhere to fibrous substrates by means of a ruthenium red-staining capsule (7, 10). This adhesion could in part be due to active-site binding of cellulases (or additional enzymes [3]) carried on the capsule when cultures are grown with PPA. One ecological advantage gained by this arrangement is that enzymatic action produces products very close to the bacterial surface and may enable the capture of a greater fraction of these products by minimizing the distance between them and the site of uptake. Since PPA-deprived cultures of *R. albus* 8 had only very low amounts of cell-associated enzymes (and no capsule), the cells from these cultures may not adhere to fibrous substrates or gain the proximity advantage discussed above.

Mediation of cell-fiber adhesion is not the only advantage gained by attachment of muralytic (plant cell wall-degrading) enzymes to the bacterial cell surface. This attachment also prevents the diffusional loss of metabolically expensive protein, which may even make possible the synthesis of the larger and presumably more efficient (see below) enzyme complexes of *R. albus*. Smaller enzymes diffusing into the surrounding medium would seem to be a less effective means of providing usable products to the enzyme-elaborating cell, especially in the rumen, where the dense population increases the competition for soluble carbohydrate (4).

Enzyme attachment and cell adhesion cannot account for all of the stimulatory effect of PPA in the cellulose cultures. Growth did not correlate with total PMC-cellulase production, since the PMC cultures without PPA produced two- to threefold-greater amounts of total PMC-cellulase activity than did their PPA-grown counterparts (Table 1); yet, they required six- to eightfold longer for growth. This implies that insofar as growth was concerned, the aggregated enzymes produced by PPA-grown cells supplied usable carbohydrate more rapidly than did the small, soluble enzymes. The greater effectiveness of the aggregates is probably due to a specific organization of components within these structures, as no additional proteins were produced by PPA-grown cultures. It seems reasonable to postulate that the organization of these aggregates is especially important in the rapid digestion of the crystalline portions of cellulose. Consistent with this postulate, the ability of *R. albus* SY3 to attack more highly ordered cellulose substrates correlated with the amount of capsule surrounding the cells (13). Since cotton fibers contain some nonglucose monomers and some glucose linkages in addition to the β1,4 glucosidic bond, it is also possible that the greater effectiveness of the aggregates may be due to the presence of other enzymes.

Wood et al. (13) also reported marked effects of various culture conditions on the cellulases produced by *R. albus* SY3. Grown in medium containing rumen fluid, SY3 cultures contained chiefly cell-bound cellulase activity, and the extracellular enzyme was of high molecular weight. They, too, suggested it was derived from cell-bound enzyme. In semidefined medium, SY3 produced only low-molecular-weight

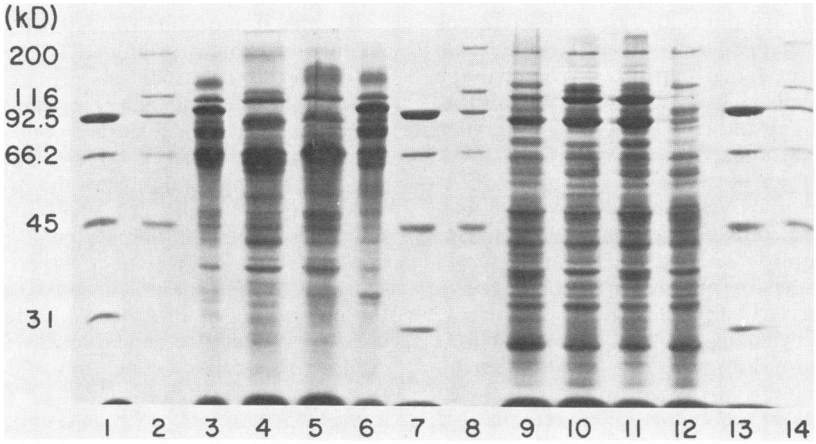

FIG. 4. Results of SDS-polyacrylamide gel electrophoresis of extracellular and cellular proteins from the four cultures. Lanes 1, 7, and 13, Bio-Rad low-molecular-mass standards (phosphorylase B, 92.5 kD; bovine serum albumen, 66.2 kD; ovalbumin, 45 kD; and carbonic anhydrase, 31 kD). Lanes 2, 8, and 14, Bio-Rad high-molecular-mass standards (myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase B, 92.5 kD; bovine serum albumen, 66.2 kD; and ovalbumin, 45 kD). Lanes 3 to 6, Extracellular protein samples from cellobiose-grown PPA-deprived culture (lane 3), cellobiose-grown culture with 25 μM PPA (lane 4), cellulose-grown culture with 25 μM PPA (lane 5), cellulose-grown cuture without PPA (lane 6). Lanes 9 to 12, Cellular protein samples from cellobiose-grown PPA-deprived culture (lane 9), cellobiose-grown culture with 25 μM PPA (lane 10), cellulose-grown culture with 25 μM PPA (lane 11), and cellulose-grown culture without PPA (lane 12).

cellulases. Since rumen fluid normally contains relatively high concentrations of PPA (5, 11), the results of Wood et al. can be explained by differences in the PPA content of their media.

In contrast to the easy extraction of the large enzyme complex from cells that was reported by Wood et al. for SY3, the cell-bound cellulase of strain 8 was only partially extractable by the treatments indicated in Table 2. This may represent a strain difference.

The similarity in the amount of total protein in all the cultures reported in Table 1 was unexpected. Any proteases in the cell must occur in low concentrations, be extremely labile, or be ineffective against other cell proteins. It was thought that maintenance energy requirements would be much greater in the slow-growing culture on cellulose without PPA, but evidently any difference in maintenance requirement was less than the variability in protein yield. The results indicate that any maintenance requirement constitutes a fixed fraction of the total metabolism regardless of culture conditions.

The PPA-depleted cultures could be transferred to unsupplemented PMC medium and still exhibit the same slow growth during several sequential transfers of the month-old cultures. In contrast, the stock cellulose cultures with PPA, with rapid growth and most of the cellulose gone within 24 h, were nonviable after 3 days of incubation. If maintenance energy is a fixed fraction, the cost to the fast-growing cultures is, in absolute terms, six to eight times greater per unit of time than in the slow-growing cultures. The cells in the fast-growing cultures are in a physiological state requiring more energy per unit time for maintenance and die quickly when substrate is exhausted, whereas the slow-growing cells, in a different physiological state, require much less energy and are able to glean sufficient substrate to retain viability over a longer period.

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