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3-Phenylpropanoic Acid Improves the Affinity of *Ruminococcus albus* for Cellulose in Continuous Culture

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A continuous-culture device, adapted for use with solid substrates, was used to evaluate the effects of 3-phenylpropanoic acid (PPA) upon the ability of the South African strain *Ruminococcus albus* Ce63 to ferment cellulose. Steady states of fermentation were established with a dilution rate of 0.17 h^{-1}, and the extent and volumetric rates of cellulose fermentation were determined over four consecutive days. When the growth medium contained no additions (control), 25 μM phenylacetate alone, 25 μM PPA alone, or 25 μM each of phenylacetate and PPA, the extent of cellulose hydrolysis was determined to be 41.1, 35.7, 90.2, and 86.9%, respectively, and the volumetric rate of cellulose hydrolysis was 103.0, 97.9, 215.5, and 230.4 mg $liter^{-1}$ h^{-1}, respectively. To evaluate the effect of PPA availability on affinity for cellulose, the values for dilution rate and extent of cellulose hydrolysis were used in combination with values for maximum specific growth rate determined from previous studies of growth rates and kinetics of cellulose hydrolysis. The findings support the contention that PPA maintains a competitive advantage for *R. albus* when grown in a dynamic, fiber-rich environment.

The current understanding of the involvement of 3-phenylpropanoic acid (PPA) in the fermentation of cellulose by *Ruminococcus albus* has been obtained from studies utilizing batch cultures and with strains of microorganisms isolated in the United States. The addition of PPA was found to improve cellulose hydrolysis (2, 10), alter cell morphology, and result in the production of cell-bound, high-molecular-weight forms of cellulases (9). Whereas PPA concentrations as low as 3 μM have been found to stimulate *R. albus*, concentrations as high as 25 μM do not stimulate the growth or rates of cellulose breakdown by some strains of *Ruminococcus flavefaciens* or *Butyrivibrio fibrisolvens* (8). Continuous-culture methods have recently been utilized to measure the kinetics of cellulose fermentation by rumen microorganisms (3, 5, 6; A. Kistner and J. H. Kornelius, submitted for publication). Continuous-culture techniques with solid substrates such as cellulose could indicate the effects of PPA upon the kinetics of cellulose hydrolysis and allow an assessment of the importance of PPA to the viability of *R. albus* in a dynamic, fiber-rich environment. We present here results from continuous-culture studies to support the contention that PPA greatly improves the affinity that *R. albus* possesses for cellulosic substrates.

R. albus strain Ce63 was isolated from a sheep fed a low-protein *Eragrostis teff* hay (7). Prior to experimentation and for continuous-culture studies, the isolate was grown on medium similar to the defined medium described by Hungate and Stack (2) but with the inclusion of Trypticase (E. Merck AG, Darmstadt, Federal Republic of Germany) (8), volatile fatty acid mixture (1), and trace elements (4). Whatman no. 1 filter paper (Whatman, Inc., Clifton, N.J.) was wet-pebble milled for 7 days at room temperature and added to the medium to give a final concentration of 1.5 g $liter^{-1}$. When necessary, phenylacetic acid (PAA) was included in the medium at a final concentration of 25 μM. The solid substrate fermentor utilized was essentially that described by Kistner et al. (3) but with recent modifications (Kistner and Kornelius, submitted). Two reaction vessels were employed, each approximately 250 ml in volume, and one was fitted with a peristaltic pump which delivered a sterile solution of PPA to maintain its concentration at approximately 25 μM. The flow rate was sufficiently slow so as to have no significant effect upon the dilution rate. Each of the reaction vessels was inoculated with 10 ml of a culture transferred at least three times through 10-ml volumes of the medium lacking both PPA and PAA. A dilution rate of 0.17 h^{-1} for both solids and liquid was established, maintaining a 98% turnover of the reaction vessel contents every 24 h. Steady state was attained, and samples from the medium reservoir and fermentation vessels were then collected on 4 consecutive days. Cellulose solubilization was determined by measuring the dry-matter disappearance of cellulose from reaction vessel contents relative to the concentration of cellulose present in the medium reservoir. Cellulose was collected onto preweighed polypropylene filters (Sartorius brand, Gottingen, Federal Republic of Germany) and washed with three 15-ml volumes of sterile, deionized water. The calculated values for the extent of cellulose solubilization were used to determine the volumetric rates of cellulose solubilization as a function of the dilution rate and treatments imposed.

The inclusion of PPA in the growth medium dramatically increased the extent and volumetric rate of cellulose hydrolysis, whereas the addition of PAA had no beneficial effects (Table 1). The more amorphous nature of the cellulosic substrate used here as compared with that of previous studies (5, 6) facilitated both the greater extent of cellulose fermentation observed and the greater dilution rates attained (Table 1). The medium was sufficiently well buffered so that pH could not be considered to have influenced cellulolytic activity. Figure 1 illustrates that concomitant with the stimulation of cellulolytic activity on PPA addition, there was an increase in the number of bacterial cells found attached to

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TABLE 1. Effect of PAA and PPA on cellulolysis by *R. albus* Ce63 grown in solid substrate fermentors

Treatment	Dilution rate (h^{-1})	Fluid pH	Cellulose degradation (%)	Volumetric rate (mg $liter^{-1}$ h^{-1})
Control	0.170	6.66	41.1	103.0
PAA	0.170	6.68	35.7	97.9
PPA	0.164	6.57	90.2	215.5
PAA + PPA	0.165	6.58	86.9	230.4

cellulose particles. However, the degree by which overall microbial yield was improved by PPA availability was not determined.

An assessment of how PPA availability altered the affinity of the bacterium for its substrate was calculated from the following equation adapted from Veldkamp (11). Values for maximum specific growth rates (μ_{max}) were chosen on the basis of growth rates previously determined in similar studies by using the same device with cellulolytic rumen microorganisms (Kistner and Kornelius, submitted): $K_s = s/[D/(\mu_{max} - D)]$. The affinity constant (K_s) for the different bacterial populations was defined as the concentration of cellulose required to support 0.5 μ_{max} where s is the steady-state concentration of residual cellulose at dilution rate D. Previous continuous-culture studies with *R. albus* using cellulose (5) showed that the conversion of cellulose to soluble sugars was the growth-limiting step because soluble sugars were negligible in culture fluids for all retention times of solids tested. Thus, cellulose per se can be considered the growth-limiting substrate. The results of the calculations are illustrated in Fig. 2. In cultures which did not contain PPA, the relative increase in the amount of cellulose required to satisfy K_s as μ_{max} increased was almost five times greater than that for cultures which contained PPA. Further, for any chosen concentration of cellulose, there were great differ-

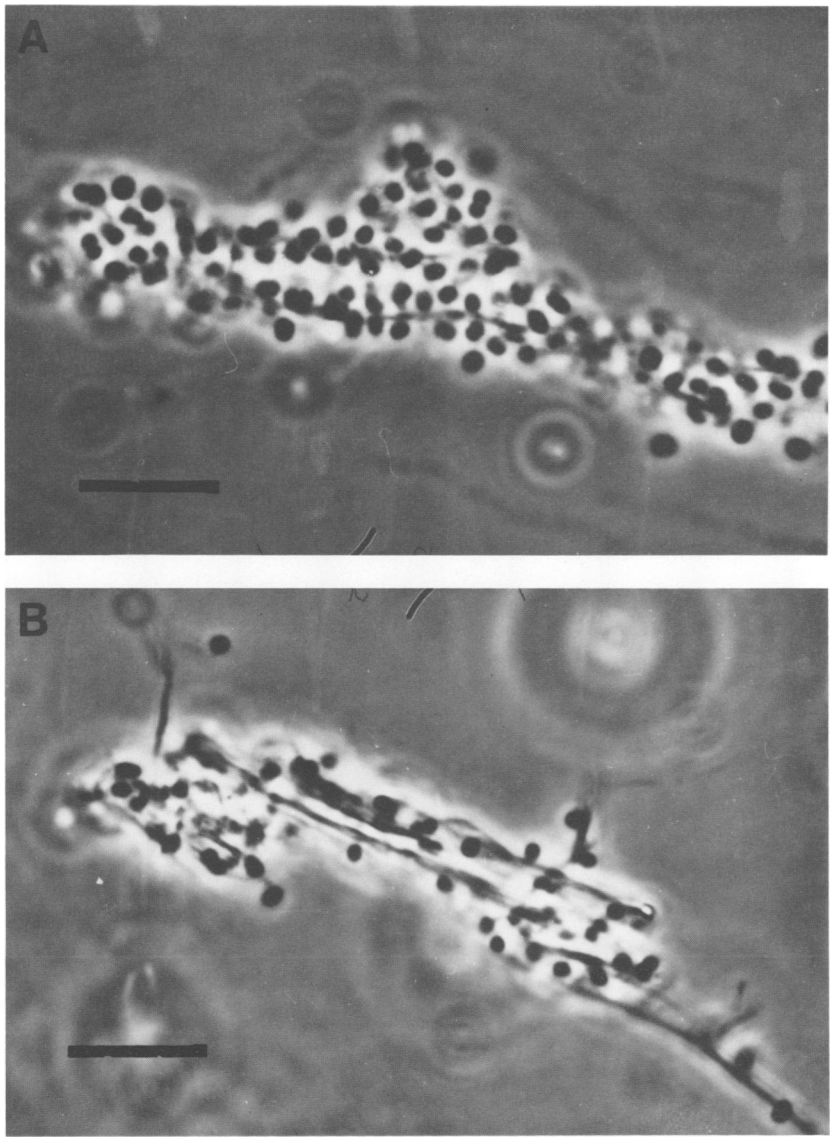

FIG. 1. Phase-contrast photomicrographs illustrating the effect upon cell density associated with cellulose in continuous culture when PPA is either present (A) or absent (B). Bar, 5 μm.

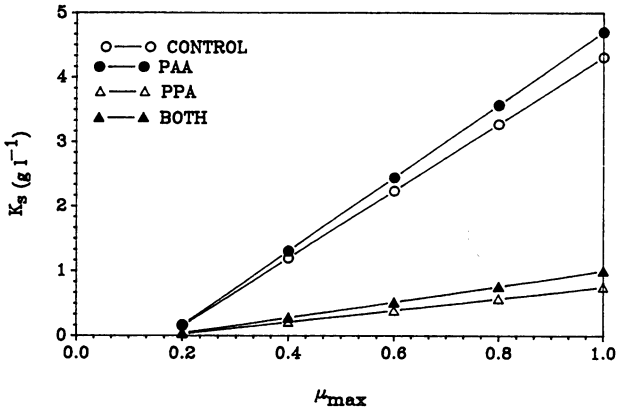

FIG. 2. Relationship between estimated μ_{max} values and K_s for *R. albus* Ce63 when grown in the presence or absence of PPA, PAA, or both.

ences in the estimated μ_{max} values, which were also dependent upon the addition of PPA. That cellulose breakdown by *R. albus* follows first-order kinetics has been shown previously with both continuous-culture (5) and batch culture (8) experiments. The explanations given for such findings (6) are consistent with the assumption that the V_{max} of total cellulase activity within each treatment does not change if the growth rate of the bacterium is changed. Thus, the calculated K_s values reflect the affinity of the bacterium for cellulose was improved by PPA addition. This may be the result of changes in either the adherence capabilities of the bacterium, the alteration of the assembly of enzymes so that a more efficient conversion of insoluble substrate into metabolizable energy proceeds, or both. Considering that cellulases are predominantly cell bound once PPA is included in the growth medium (2, 9), the adherence of *R. albus* to its substrate could become a more important parameter affecting the in vivo rate and extent of cellulose hydrolysis. The data presented in this study would support the contention that the adherence capabilities of *R. albus* were improved by the availability of PPA. By increasing affinity for cellulose, PPA improves the competitiveness of *R. albus* for its substrate in a dynamic, fiber-rich environment, and hence it has major role in ruminal fiber degradation. These continuous-culture studies reinforce the essential role of microbial diversity in the rumen, i.e., that provision of nutrients such as PPA facilitates the conversion of cellulosic materials into metabolic energy.

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